THE MULTIFACETED ROLES OF LIPIDS IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STATES

EDITED BY: Simona Lobasso, Jean-Marc A. Lobaccaro and Roberto Angelini

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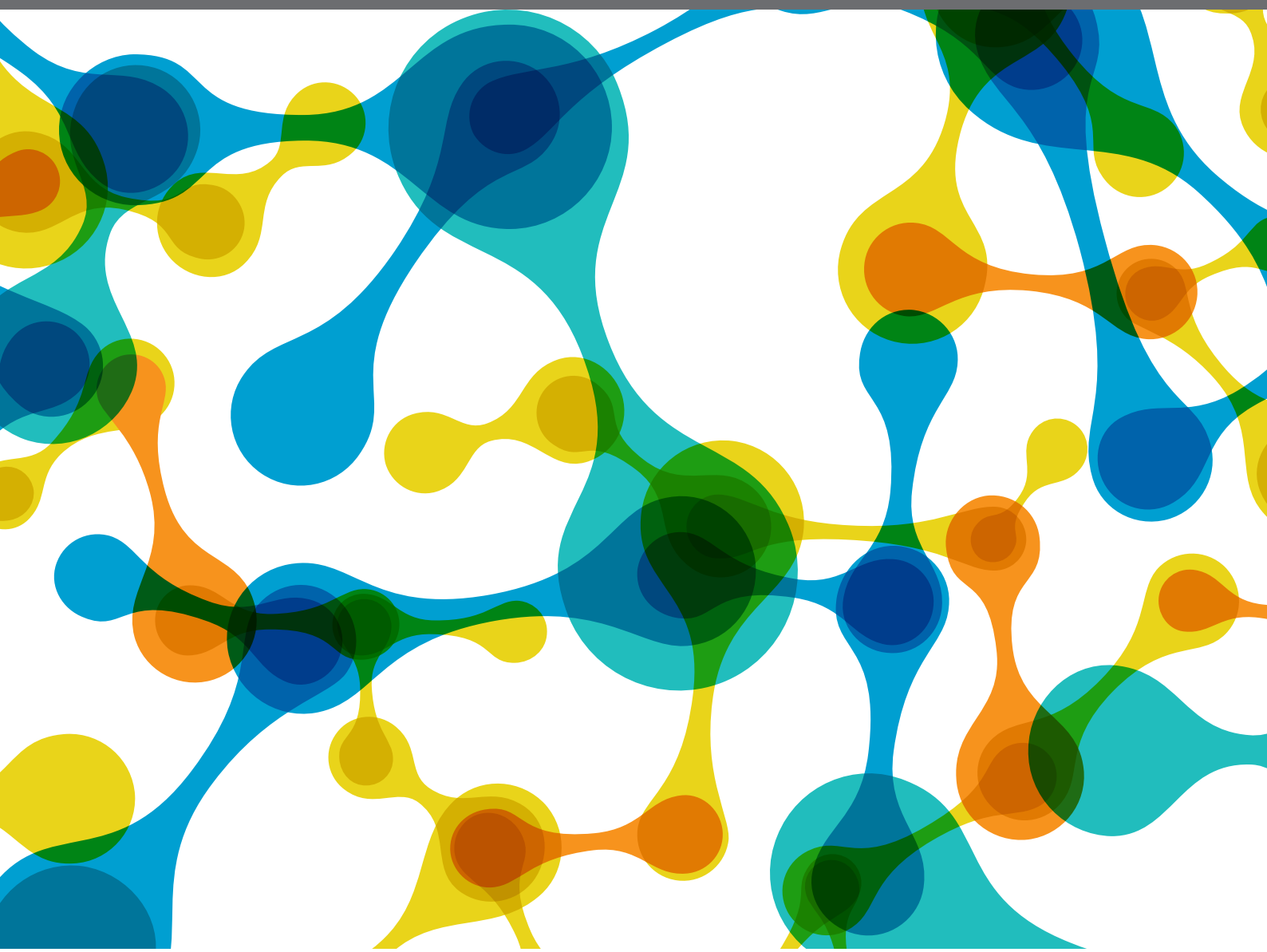

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THE MULTIFACETED ROLES OF LIPIDS IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STATES

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Editorial: The multifaceted roles of lipids in physiological and pathophysiological states

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Editorial on the Research Topic
The multifaceted roles of lipids in physiological and pathophysiological states

Lipids have been first described during 18th century and since then they have been described in all living tissues. The chemistry of lipids started when the French chemist Poultier de la Salle (1719–1788) for the first time isolated cholesterol crystals from bile. Although Poultier's work was never published, his disciples had quoted it later.

Recently, the advance in technology further improved the ability to characterize their highly variable structure and function. Almost 250 years later, this special volume of Frontiers in Physiology entitled "*The multifaceted roles of lipids in physiological and pathophysiological states*" aims to cover the last advances in lipid physiology. Because the human lipidome is made of thousands of lipid molecules, it is no wonder that their different chemical structures may exert an enormous variety of biological functions. These include energy production and membrane structural scaffold, sorting and regulation of membrane proteins, cellular signaling and vesicle trafficking. Lipidomics has been developed to study qualitatively and quantitatively these multifaceted molecules. This OMICS technology is a relatively young branch of analytical chemistry as well as an interdisciplinary field of study involving biochemistry and biophysics, applied mass spectrometry, complex statistical analyses, and miniaturization of the assays, which needs increased analytical standardization, especially in the quantification of individual lipid species. In addition, fine alterations of lipid composition can lead to a wide spectrum of human pathologies, ranging from cancer to metabolic, cardiovascular and neurodegenerative diseases. Enlightening this complexity, the various articles pinpoint some of these facts. The readers will thus find a Research Topic of articles on lipid functions ranging from oxysterols to cardiolipin, from fatty acids derivatives to protein lipidation and beyond.

Indeed, Wiley et al. describe new methods to assess activity of enzymes responsible for biosynthesis and degradation of endocannabinoid and related lipids in various mouse mucosal tissues. Griffiths and Wang suggest that oxysterols could rapidly act as a paracrine version of free cholesterol, mainly for resistance to microbial pathogens. Bozelli et al. enlighten the links between plasmalogens, common glycerophospholipids, and chronic inflammatory pathologies, and how they can be used to prevent inflammation. Engel et al. stress that determination of lipid biomarkers such as lysophosphatidylcholine needs a solid expertise. This point is crucial especially whether this biomarker is used to define pathological processes such as infertility, metabolic disorders, and cancers. Elkes et al. point out that diet supplementation with linoleic acid could ameliorate the effects of tafazzin deficiency in an animal model of the cardiomyopathy Barth syndrome. The link between lipids and immune cell physiology is ascertain by Zhang et al. work. It describes the lipid droplets as a "central hub" connecting metabolism and inflammation, and not only as lipid "bags". Lobasso et al. report a comprehensive lipid analysis of exosomes secreted from melanoma cells having different metastatic behavior by a lipidomic approach. Hamsanathan and Gurkar review the current knowledge on lipid metabolism and cellular lipids during senescence. They clearly identify specific lipids such as 15d-PGJ2 as a biomarker of senescent cell removal (senolysis). The authors also define C16:0 ceramide level as a prognosis marker of functional decline. Likewise, Dai et al. describe that lifespan could be regulated by phosphatidylethanolamine, phosphatidylserine and cardiolipin levels, even though the precise mechanism is still unknown. Lipids may also be associated to proteins to modify their activity. This point is exemplified by Thomas et al. with Ghrelin, an orexigenic hormone that presents a unique octanoyl modification within its peptide sequence. The authors discuss the role of Ghrelin acylation for the brain physiology and the possible consequences of the acylation for cognition and neuropathology.

Ralph-Epps et al. summarise the important role of cardiolipin in Barth syndrome using the yeast model *Saccharomyces cerevisiae*. Beside an understandable alteration of the mitochondrial bioenergetics, the authors highlight that tafazzin defects also impair iron and calcium metabolism.

Altogether, this Research Topic, even though not exhaustive, offers various examples of successful strategies for dissecting out the multifaceted roles of lipids in physiology and pathology. We believe that this volume could be a springboard for other Research Topics that focus on lipid measurements, lipid homeostasis and their role in pathologies.

Author contributions

SL, J-MAL, and RA equally wrote, reviewed and edited this editorial.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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METHODS
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UPLC-MS/MS Method for Analysis of Endocannabinoid and Related Lipid Metabolism in Mouse Mucosal Tissue

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The endocannabinoid system is expressed in cells throughout the body and controls a variety of physiological and pathophysiological functions. We describe robust and reproducible UPLC-MS/MS-based methods for analyzing metabolism of the endocannabinoids, 2-arachidonoyl-*sn*-glycerol and arachidonoyl ethanolamide, and related monoacylglycerols (MAGs) and fatty acid ethanolamides (FAEs), respectively, in mouse mucosal tissues (i.e., intestine and lung). These methods are optimized for analysis of activity of the MAG biosynthetic enzyme, diacylglycerol lipase (DGL), and MAG degradative enzymes, monoacylglycerol lipase (MGL) and alpha/beta hydrolase domain containing-6 (ABHD6). Moreover, we describe a novel UPLC-MS/MS-based method for analyzing activity of the FAE degradative enzyme, fatty acid amide hydrolase (FAAH), that does not require use of radioactive substrates. In addition, we describe *in vivo* pharmacological methods to inhibit MAG biosynthesis selectively in the mouse small-intestinal epithelium. These methods will be useful for profiling endocannabinoid metabolism in rodent mucosal tissues in health and disease.

Keywords: UPLC-MS/MS, endocannabinoids, lipid metabolism, enzyme kinetics, fatty acid amide hydrolase, monoacylglycerol lipase, diacylglycerol lipase, alpha/beta hydrolase domain containing 6

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INTRODUCTION

The endocannabinoid (eCB) system is expressed in cells throughout the body and consists of lipid signaling molecules including the primary eCBs, 2-arachidonoyl-*sn*-glycerol (2-AG) and arachidonoyl ethanolamide (anandamide, AEA), their biosynthetic and degradative enzymes, and the cannabinoid receptors [cannabinoid receptor subtype-1 (CB_1R) and subtype-2 (CB_2R),

Abbreviations: FFA, free fatty acid; NAAA, *N*-acylethanolamine acid amidase; SIR, selected ion reading; FAE, fatty acid ethanolamide; MAG, monoacylglycerol; DGL, diacylglycerol lipase; MGL, monoacylglycerol lipase; ABHD6, alpha/beta hydrolase domain containing 6; ABHD12, alpha/beta hydrolase domain containing 12; FAAH, fatty acid amide hydrolase; THL, tetrahydrolipostatin; PEG, polyethylene glycol; 2-AG, 2-arachidonoyl-*sn*-glycerol; 2-DG, 2-docosohexaenoyl-glycerol; 2-PG, 2-palmitoyl-glycerol; 2-OG, 2-oleoyl-glycerol; 2-LG, 2-linolenyl-glyceroly; AEA, anandamide; OEA, oleoylethanolamide; DHEA, docosohexaenoylethanolamide; PA, palmitic acid; eCB, endocannabinoid; CB_1R, cannabinoid receptor subtype-1; CB_2R, cannabinoid receptor subtype-2.

and possibly others] (Devane et al., 1987, 1992; Kaminski et al., 1992; Mechoulam et al., 1995; Piomelli, 2003; Pertwee, 2015; see **Figure 1**). ECBs are produced upon cellular activation and synthesized from lipid precursors found in the plasma membrane of cells (DiPatrizio, 2021). 2-AG and AEA activate the same cannabinoid receptors; however, their biosynthesis and degradation are controlled by distinct enzymatic pathways. Diacylglycerol lipase (DGL) hydrolyzes distinct diacylglycerol precursors and generates 2-AG and other related monoacylglycerol (MAG) species including 2-docosohexaenoylglycerol (2-DG), 2-pamitoylglycerol (2-PG), 2-oleoylglycerol (2-OG), and 2-linoleoylglycerol (2-LG) (Ghafouri et al., 2004; Alexander and Kendall, 2007; DiPatrizio, 2021). These MAGs are degraded via monoacylglycerol lipase (MGL) into free fatty acids and glycerol (DiPatrizio, 2021). Furthermore, alpha/beta hydrolase domain containing-6 (ABHD6) and -12 (ABHD12) are capable of degrading MAGs, including 2-AG, and contribute to total MAG degradation in the brain (Blankman et al., 2007; Marrs et al., 2010; DiPatrizio, 2021).

In contrast to MAGs, fatty acid ethanolamides (FAEs) are synthesized from *N*-acylphosphatidylethanolamine (NAPE), which is produced by activity of *N*-acyltransferase (NAT) in a Ca^{2+}- and cAMP-dependent manner (Di Marzo et al., 1994; Cadas et al., 1996, 1997; Hussain et al., 2017; Tsuboi et al., 2018). NAT transfers a fatty acid (i.e., arachidonate) from the *sn*-1 position of a phospholipid to the amino group of the phosphatidylethanolamine to produce distinct NAPEs (Di Marzo et al., 1994; Cadas et al., 1996, 1997; Hussain et al., 2017; Tsuboi et al., 2018). NAPE is then hydrolyzed via *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) to produce FAEs that include AEA, oleoylethanolamide (OEA), and docosohexaenoylethanolamide (DHEA) (DiPatrizio, 2021). These FAEs are subsequently degraded by fatty acid amide hydrolase (FAAH) into free fatty acids and ethanolamine (Cravatt et al., 1996; Wei et al., 2006). Furthermore, *N*-acylethanolamine acid amidase (NAAA) also hydrolyzes some FAEs, including palmitoylethanolamide (PEA), and has been identified primarily in endosomal-lysosomal compartments of adaptive and innate immune cells (Tsuboi et al., 2007, 2018; Piomelli et al., 2020).

Direct pharmacological manipulation of CB_1R activity with, for example, globally-acting antagonists/inverse agonists (i.e., rimonabant) reduces body weight and improves a host of metabolic parameters in human obesity; however, these drugs reach the brain and can lead to psychiatric side effects that preclude their use in the clinic for the treatment of metabolic disease (Christensen et al., 2007). In contrast to directly targeting cannabinoid receptors, pharmacological manipulation of enzymes responsible for the biosynthesis or degradation of eCBs may provide a safe therapeutic strategy for treatment of a variety of disorders. Accordingly, reliable methods for identifying tissue-specific changes in eCB turnover is critical for informing development of therapeutics that target metabolism of eCBs. Several existing methods for detecting changes in enzyme activity rely on fluorogenic or chromogenic enzyme substrates and products which, while highly effective, are not optimal for monitoring activity of a variety of enzymatic reactions (Sun et al., 2018; de Rond et al., 2019). The introduction of a label, including non-natural fluorogenic residues near the carboxyl- or amino-terminal side of the substrate, can significantly alter its conversion to product by the enzyme of interest (Su et al., 2006). Therefore, the use of label-free assays provides significant improvements in accurately determining enzymatic activity. Furthermore, nearly all enzymatic reactions involve a change in substrate mass, therefore mass spectrometry (MS) is ideal for quantitation of enzyme activity. Coupling a chromatographic step (i.e., liquid chromatography) to MS provides physical and temporal separation of analytes and significantly increases sensitivity. These advantages have motivated work to develop LC-MS/MS based methods for analyzing enzyme activity (Ohira et al., 2018).

Here, we describe methods using ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) to assess activity of enzymes responsible for biosynthesis and degradation of eCBs and related lipids in distinct mouse mucosal tissues. These methods are optimized for quantitation of the rate of metabolism of eCBs by DGL, MGL, ABHD6, and FAAH. Moreover, novel methods are described for measuring FAAH activity that does not require use of radioactive compounds as substrates (Fu et al., 2007; Dainese et al., 2020).

MATERIALS AND METHODS

Chemicals and Compounds

The following compounds were used as substrates: dinonadecadienoin (19:2 DAG, Nu-Chek Prep, Waterville, MN, United States) for the DGL assay, non-adecadienoin (19:2 MAG; Nu-Chek Prep) for the MGL and ABHD6 assays, and [$^2H_4$]-palmitoyl-ethanolamide ([$^2H_4$]-PEA, Cayman Chemical, Ann Arbor, MI, United States) for FAAH assays. The following compounds were used as internal standards for both lipid extracts and enzyme assays: [$^2H_5$] 2-AG (Cayman Chemical, Ann Arbor, MI, United States) for lipid extracts and the DGL assay, heptadecanoic acid (17:1 FFA; Nu-Chek Prep) for the MGL/ABHD6 and FAAH activity assays, [$^2H_4$]-OEA (Cayman Chemical, Ann Arbor, MI, United States) and [$^2H_4$]-AEA (Cayman Chemical, Ann Arbor, MI, United States) for lipid extracts. The following chemicals were used as inhibitors for enzyme assays: tetrahydrolipstatin (THL) (Cayman Chemical, Ann Arbor, MI, United States) for DGL inhibition, JZL 184 (Cayman Chemical, Ann Arbor, MI, United States) for MGL inhibition, WWL 70 (Cayman Chemical, Ann Arbor, MI, United States) for ABHD6 inhibition, and URB597 (Cayman Chemical, Ann Arbor, MI, United States) for FAAH inhibition. Commercially available substrates or internal standards, (i.e., odd-numbered fatty acid chains, deuterated molecules) were used across assays due to their low cost and to ensure that detection of products of reactions were selective for their unique substrates versus endogenously produced molecules that can interfere with detection and quantitation of activity.

Tissue Harvest and Preparation for Enzyme Assays

Adult C57BL/6J male mice (Jackson Laboratories) were maintained with *ad libitum* access to food and water, and were anesthetized using isoflurane prior to tissue harvest.

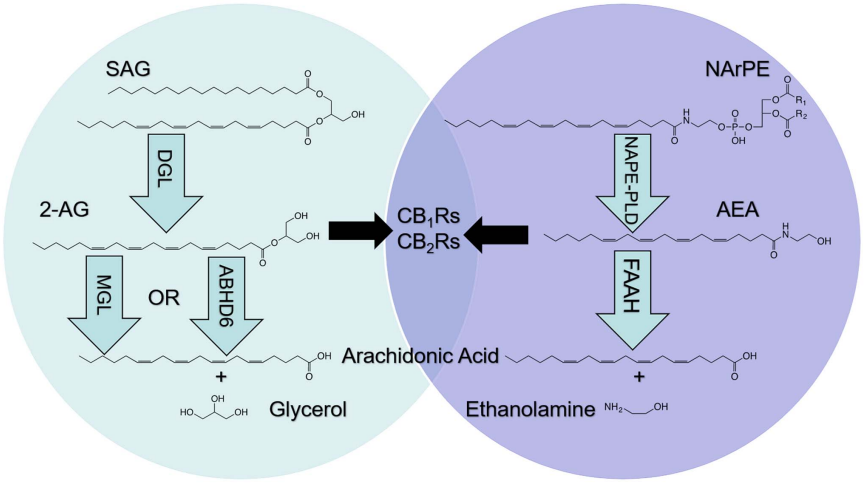

FIGURE 1 | Metabolism of endocannabinoids (eCBs). The eCBs, 2-arachidonoyl-*sn*-glycerol (2-AG) and arachidonoyl ethanolamide (AEA), activate cannabinoid receptor subtype-1 (CB_1Rs) and subtype-2 (CB_2Rs) located in cells throughout the body; however, metabolic pathways are not shared. 2-AG is produced following hydrolysis of the 2-AG precursor, 1, stearoyl,2-arachidonoyl-*sn*-glycerol (SAG), by diacylglycerol lipase (DGL). 2-AG is degraded by monoacylglycerol lipase (MGL) and alpha/beta hydrolase domain-6 (ABHD6) into arachidonic acid (AA) and glycerol (left). AEA is produced following hydrolysis of the AEA precursor, *N*-arachidonoylphosphatidylethanolamide (NArPE), by *N*-acylphosphatidylethanolamide phospholipase D (NAPE-PLD). AEA is degraded by fatty acid amide hydrolase (FAAH) into AA and ethanolamine (right).

Proximal small-intestinal (jejunum) and lung were removed and rinsed in ice-chilled 1× PBS (pH = 7.0). Jejunum was opened longitudinally, and gently washed. Glass slides were used to scrape the intestinal epithelium layer, placed on dry ice, and then snap frozen in liquid N_2. Lung tissue was removed, rinsed in ice-chilled 1× PBS (pH = 7.0), and snap frozen using liquid N_2. Samples were stored at −80°C until processing. All procedures met the United States National Institute of Health guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC Protocol 20200022) of the University of California, Riverside.

Oral Gavage and Tissue Harvest

Adult C57BL/6J male mice were food-deprived for 24 h prior to harvest with *ad libitum* access to water. To prevent coprophagia, animals were maintained on elevated wire-bottom cages for a 72-hour acclimation period and during the 24-hour food deprivation. One hour prior to harvest, mice received an oral gavage (100 μL of 10 mg/mL) of the DGL inhibitor, THL, in polyethylene glycol (PEG) or PEG alone as control. Jejunum intestinal epithelium and lungs were harvested as described above (see section "Tissue Harvest and Preparation for Enzyme Assays").

Protein Preparation

Approximately 100 mg of intestinal epithelium or 50 mg of lung tissue was weighed and placed into 2 mL of chilled 50 mM Tris–HCl, 320 mM sucrose buffer (pH = 7.5). Samples were blade homogenized at 15,000 + rpm for 10–20 s. The blade was twice cleaned with chilled water and acetone washes between samples. Homogenized samples were used for assay-specific protein isolations described below.

DGL, MGL, and ABHD6 Protein Isolation

Homogenized samples were centrifuged at 800 × *g* for 15 m at 4°C. The supernatant was collected in a 2.0 mL centrifuge tube and sonicated twice for 10 s. Samples underwent two sequential freeze thaw cycles using liquid N_2. Samples were again centrifuged at 800 × *g*, 15 min, 4°C and the supernatant was collected. Total protein was then quantified via BCA assay and normalized between all samples. Protein isolations for each assay and each tissue were performed separately.

FAAH Protein Isolation

Homogenized samples were centrifuged at 21,100 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellets were resuspended in 750 μL of chilled 1x PBS (pH = 7.0). To ensure homogenous solution, lung samples were further homogenized using a sonic dismembranator using 8–10 root mean square (RMS) Watts of output power. Total protein was then quantified via BCA assay and normalized between all samples. Alternative protein isolation methods were required for FAAH isolation due to its subcellular localization to membranes of cytoplasmic organelles (Gulyas et al., 2004).

Enzyme Assays

MAG Biosynthetic Enzyme Activity Assay

Substrate solutions were prepared by drying stock 19:2 DAG (20 nmol/reaction) under N_2 steam (99.998% pure) and reconstituted in a solution of 50 mM Tris–HCl with 0.2% Triton x-100 (pH = 7.0). This solution was bath sonicated for 60 min at room temperature while protein samples were prepared. Normalized protein samples (100 μL) from jejunum epithelium homogenates or lung homogenates were incubated at room temperature with the MGL inhibitor, JZL 184 (6 μM) for

10 min to ensure the product of interest was not metabolized (Long et al., 2009). In addition, the ABHD6 inhibitor, WWL 70 (10 μM), was added to lung protein isolates for the 10-minute room temperature incubation to ensure the product of interest was not metabolized (Tchantchou and Zhang, 2013). Dose-inhibition experiments included addition of the DGL inhibitor, THL [albeit not selective for DGL, see Hoover et al. (2008); DiPatrizio et al. (2015)]. Next, 100 μL of DGL substrate solution was added to normalized protein samples (100 μL; 200 μL final volume) and incubated in water bath at 37°C for 30 min. The reaction was stopped by the addition of 1.0 mL of chilled methanol containing 25 pmol of the internal standard [$^2H_5$]-2-AG. The products of the reaction were extracted via lipid extraction methods (see section "Lipid Extraction for Enzyme Assays") and quantified via UPLC-MS/MS (see section "Quantitation of MAG Biosynthetic Enzyme Activity Assay Products").

MAG Degradative Enzyme Activity Assays

Substrate solutions were prepared by drying stock 19:2 MAG (50 nmol/reaction) under N_2 steam and adding fatty acid free BSA (0.25%) and stock 50 mM Tris–HCl (pH = 8.0). The MGL substrate solution was then sonicated for 60 min while protein samples were prepared. Dose-inhibition experiments included a 10-min pre-incubation of protein samples at room temperature with varying concentrations of either the selective MGL inhibitor, JZL 184, and/or the selective ABHD6 inhibitor, WWL 70, prior to addition of the substrate solution. MGL substrate solution was added to normalized protein (400 μL; 500 μL final volume) samples and incubated in a water bath at 37°C for 10 min (jejunum protein) or 30 min (lung protein). The reaction was stopped using 1.0 mL of chilled methanol containing the internal standard 17:1 FFA (5 nmol/reaction) and placed on ice. The products of the reaction were extracted via lipid extraction methods (see section "Lipid Extraction for Enzyme Assays") and quantified via UPLC-MS/MS (see section "Quantitation of MAG Degradative Enzyme Activity Assay Products").

FAE Degradative Enzyme Activity Assay

Substrate solutions were prepared by drying stock [$^2H_4$]-PEA (5 nmol/reaction) under N_2 steam and adding fatty acid free BSA (0.25%) and stock 50 mM Tris–HCl (pH = 8.0). The substrate solution was then bath sonicated for 60 min while protein samples were prepared. Dose-inhibition experiments included a 10-minute incubation at room temperature with varying concentrations of the FAAH inhibitor URB597 (Piomelli et al., 2006) prior to incubation with the substrate. Next, 100 μL of FAAH substrate solution was added to normalized protein samples (400 μL; 500 μL final volume) and incubated at 37°C for 30 min. The reaction was stopped using 1.0 mL of methanol containing the internal standard 17:1 FFA (5 nmol/reaction) and immediately placed on ice. The products of the reaction were extracted via lipid extraction methods (see section "Lipid Extraction for Enzyme Assays") and quantified via UPLC-MS/MS (see section "Quantitation of FAE Degradative Enzyme Activity").

Lipid Extractions

Lipid Extraction for Enzyme Assays

Lipids were extracted using liquid-liquid extraction with chloroform (2.0 mL) followed by 0.8 mL 0.2-micron ultra-purified water. Samples were centrifuged (1500 × *g*, 5 min, 4°C) and the lower organic phase was collected. The samples were further purified, as previously described (Batugedara et al., 2018), via open-bed silica gel column chromatography which was washed with a 9:1 chloroform:methanol mixture to elute MAGs, FAEs, and FFAs for collection. Eluates were dried under N_2 steam (99.998% pure) and resuspended in 0.2 mL of methanol:chloroform (1:1). Products were detected and quantified via UPLC-MS/MS techniques (see section "UPLC-MS/MS Detection of Analytes").

Tissue Lipid Extraction for FAE and MAG Quantitation

Frozen tissue samples were weighed and homogenized in 1.0 mL of methanol containing the internal standards [$^2H_5$]-2-AG (500 pmol), [$^2H_4$]-AEA (1 pmol), and [$^2H_4$]-OEA (10 pmol). Lipids were extracted using chloroform (2.0 mL) prior to being washed with 1.0 mL 0.2-micron ultra-purified water. Following centrifugation (1,500 × *g*, 15 min, 4°C), the lower organic phase was collected and dried under N_2 steam (99.998% pure). A second chloroform wash (1.0 mL) was then performed followed by another centrifugation (1,500 × *g*, 15 min, 4°C) and collection of the lower phase. Samples were reconstituted in 2.0 mL of chloroform and purified via open-bed silica gel column chromatography. Columns were washed with a 9:1 chloroform:methanol mixture to elute MAGs and FAEs for collection. Collected eluates were dried under N_2 steam (99.998% pure) and resuspended in 0.2 mL of methanol:chloroform (1:1) prior to analysis via UPLC-MS/MS (see section "Quantitation of MAGs and FAEs").

UPLC-MS/MS Detection of Analytes

Data was acquired using an Acquity I-Class UPLC with direct line connection to a Xevo TQ-S Micro Mass Spectrometer (Waters Corporation, Milford, MA, United States) with electrospray ionization (ESI) sample delivery. Lipids were separated using an Acquity UPLC BEH C_{18} column (2.1 × 50 mm i.d., 1.7 μm, Waters) and inline Acquity guard column (UPLC BEH C_{18} VanGuard PreColumn; 2.1 × 5 mm i.d.; 1.7 μm, Waters), and eluted by an analyte specific gradient of water and methanol (both containing 0.25% acetic acid, 5 mM ammonium acetate). Samples were kept at 10°C in the sample manager and the column was maintained at 40°C. Argon (99.998%) was used as collision gas.

Quantitation of MAG Biosynthetic Enzyme Activity Assay Products

Analytes were eluted at a flow rate of 0.4 mL/min and gradient: 80% methanol 0.0–0.5 min, 80–100% methanol 0.5–2.5 min, 100% methanol 2.5–3.0 min, 100–80% methanol 3.0–3.1 min, and 80% methanol 3.1–4.5 min. MS/MS detection was in positive ion mode with capillary voltage maintained at 1.10 kV. Cone voltages and collision energies for respective analytes: 19:2 MAG = 18 v, 10 v; [$^2H_5$]-2-AG = 25 v, 44 v. Lipids were quantified using a stable isotope serial dilution method detecting H^+ or Na^+

adducts of the molecular ions $[M + H/Na]^+$ in multiple reactions monitoring (MRM) mode (variable amounts of product 19:2 MAG versus fixed amount of internal standard $[^2H_5]$-2-AG). Acyl migration from sn-2 to sn-1 positions in monoacylglycerols is known to occur (Stella et al., 1997; Roxana et al., 2001); thus the sum of these isoforms ($[^2H_5]$-1-AG and $[^2H_5]$-2-AG) is presented. Extracted ion chromatograms for MRM transitions were used to quantify analytes: 19:2 MAG (m/z = 386.4 > 277.2) product of DGL assay and $[^2H_5]$-2-AG (m/z = 384.3 > 93.4) as internal standard.

Quantitation of MAG Degradative Enzyme Activity Assay Products

Data was acquired using equipment described above (see section "UPLC-MS/MS Detection of Analytes") and eluted by a gradient of water and methanol (containing 0.25% acetic acid, 5 mM ammonium acetate) at a flow rate of 0.4 mL/min and gradient: 90% methanol 0.0–0.1 min, 90–100% methanol 0.1–2.0 min, 100% methanol 2.0–2.1 min, 100–90% methanol 2.1–2.2 min, and 90% methanol 2.2–2.5 min. MS detection was in negative ion mode with capillary voltage maintained at 3.00 kV. Cone voltages for non-adecadienoic acid (19:2 FFA) = 48 v and 17:1 FFA = 64 v. Lipids were quantified using a dilution series detecting deprotonated molecular ions in selected ion reading (SIR) mode (variable amounts of product 19:2 FFA versus fixed amount of internal standard 17:1 FFA). Extracted ion chromatograms for SIR masses were used to quantify analytes: 19:2 FFA (m/z = 293.2) product of MGL enzyme assay and 17:1 FFA (m/z = 267.2) as internal standard. Signal to noise ratio was >10 for all quantitated results.

Quantitation of FAE Degradative Enzyme Activity Products

Data was acquired using equipment described above (see section "UPLC-MS/MS Detection of Analytes") and using the elution protocol described above (see section "Quantitation of MAG Degradative Enzyme Activity Assay Products"). Cone voltage for palmitic acid ($[^2H_4]$-PA) = 54. Lipids were quantified using a dilution series detecting deprotonated molecular ions in SIR mode (variable amounts of product $[^2H_4]$-PA versus fixed amount of internal standard 17:1 FFA). Extracted ion chromatograms for SIR masses were used to quantify $[^2H_4]$-PA (m/z = 259.3) product of FAAH enzyme assay.

Quantitation of MAGs and FAEs

Data was acquired using equipment described above (see section "UPLC-MS/MS Detection of Analytes") and using the elution protocol described above (see section "Quantitation of MAG Biosynthetic Enzyme Activity Assay Products"). Cone voltage and collision energy for each analyte are as follows, respectively: AEA = 30 v, 14 v; $[^2H_4]$-AEA = 26 v, 16v; OEA = 28 v, 16 v; $[^2H_4]$-OEA = 48 v, 14 v; DHEA = 30 v, 50 v; 2-AG (20:4) = 30 v, 12 v; $[^2H_5]$-2-AG = 25 v, 44v; 2-DG (22:6) = 34 v, 14 v; 2-PG (16:0) = 18 v, 10 v; 2-OG (18:1) = 42 v, 10 v; 2-LG (18:2) = 30 v, 10 v. MS/MS detection was in positive ion mode and capillary voltage set at 0.1 kV. Extracted ion chromatograms were used to quantify AEA (m/z = 348.3 > 62.0), $[^2H_4]$-AEA (m/z = 352.3 > 66.1), OEA (m/z = 326.4 > 62.1), $[^2H_4]$-OEA (m/z = 330.4 > 66.0), DHEA (m/z = 372.3 > 91.0), 2-AG (m/z = 379.3 > 287.3), $[^2H_5]$-2-AG (m/z = 384.3 > 93.4), 2-DG (m/z = 403.3 > 311.1), 2-PG (m/z = 331.3 > 239.3), 2-OG (m/z = 357.4 > 265.2), and 2-LG (m/z = 355.3 > 263.3). Quantitation occurred using a stable isotope dilution method to detect protonated adducts of the ions [M + H] + in MRM mode. Acyl migration is known to occur in many MAG species following silica-gel purification, therefore the sum of 1-AG and 2-AG, 1-PG and 2-PG, 1-OG and 2-OG, and 1-DG and 2-DG are reported (Stella et al., 1997). The established lower limit of quantitation (LLOQ: signal to noise ratio >10) for 2-AG, 2-DG, 2-PG, 2-OG, and 2-LG was 0.5 pmol. The LLOQ for AEA, OEA, and DHEA was 0.008 pmol.

Statistical Analysis

Data was analyzed using GraphPad Prism7 software. Analyte specific standard curves were generated using linear regression models. All protein curves were generated using Michaelis-Menten regression models. Inhibition curves were generated using non-linear regression showing log[inhibitor] vs. normalized response. Lastly, multiple unpaired t-tests were performed on jejunum mucosa MAGs and FAEs and lung MAGs and FAEs with significance indicated by a $p < 0.05$. All values are expressed as mean ± SEM.

RESULTS

Enzyme Assay Standard Curves

Quantitation of products of all reactions were made by standard isotope dilution methods that include plotting the ratio between analyte of interest versus fixed amounts of assay-specific internal standards. Each standard curve had high coefficient of determination ($r^2 > 0.95$) indicating the actual values are close to the generated linear regression (**Figure 2**). Additionally, all analyte specific values from the enzyme assays were within the limits of the generated standard curves. These standard curves were used to determine the amount of product generated during the enzyme activity assays. Representative chromatograms including retention times and predicted SIR and MRM masses are included in **Supplementary Figure 1**.

Protein Concentration Optimization for Assaying Enzyme Activity in Jejunum Epithelium

Optimal protein concentrations for assays were determined for DGL activity (**Figure 3A**), MGL activity (**Figure 3B**), and FAE hydrolyzing activity (**Figure 3C**) in mouse jejunum mucosal tissue. All protein curves had a high coefficient of determination ($r^2 \geq 0.93$). Increasing levels of isolated protein from jejunum mucosa (1.56–50 μg) for the DGL activity assay (**Figure 3A**) led to associated increases in product recovery (0.000029 ± 0.000025–0.06 ± 0.002 nmol/min 19:2 MAG). MGL activity in the jejunum mucosa (**Figure 3B**) also indicated that as isolated protein (1.56–50 μg) increased, the amount of product

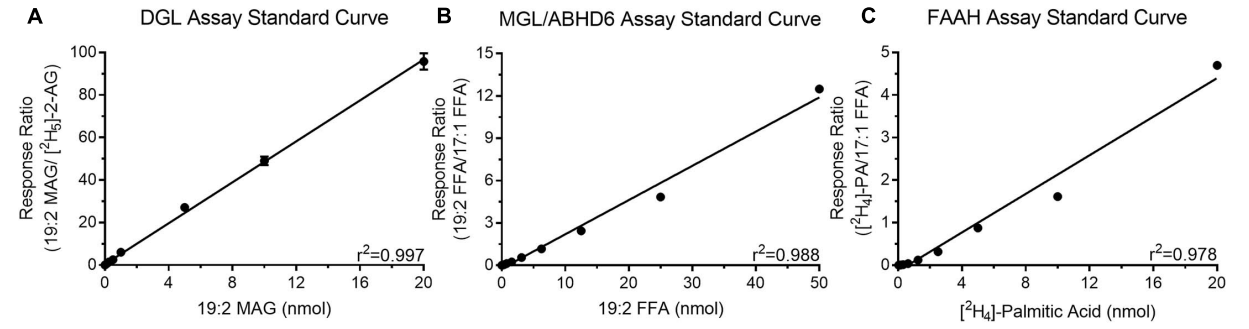

FIGURE 2 | Standard curves for quantitation of the products generated during the DGL **(A)**, MGL/ABHD6 **(B)**, and FAAH **(C)** activity assays.

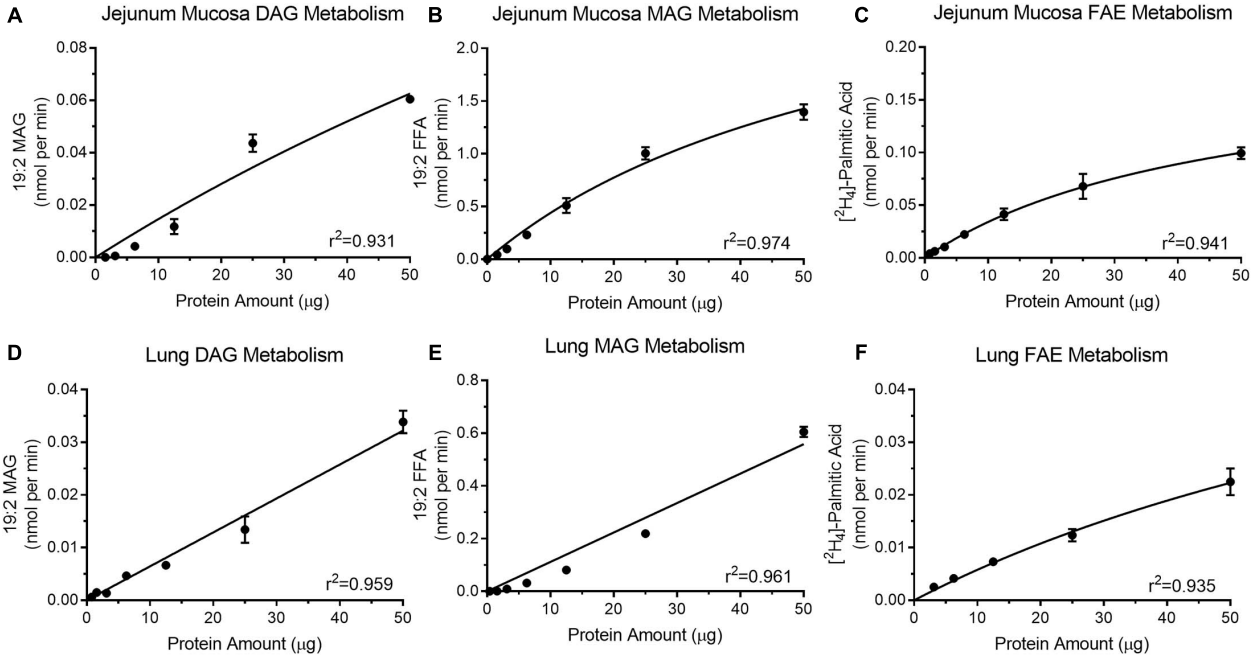

FIGURE 3 | Isolated protein from mouse jejunum mucosa display increases in product recovery as protein amount increases for DGL activity **(A)**, MGL activity **(B)**, and FAAH **(C)**. Mouse lung protein isolates exhibit increases in product recovery as the amount of protein increased for DGL **(D)**, MGL **(E)**, and FAAH **(F)**.

recovered also increased (0.042 ± 0.005–1.396 ± 0.074 nmol/min 19:2 FFA). Lastly, FAE hydrolyzing activity in the mouse jejunum (**Figure 3C**) displayed a similar trend with increasing protein (0.78–50 μg) resulting in increased product recovery (0.004 ± 0.00013–0.099 ± 0.006 nmol/min [$^2H_4$]-PA).

Protein Concentration Optimization for Quantitating Enzyme Activity in Lung

Diacylglycerol lipase activity (**Figure 3D**), MGL/ABHD6 activity (**Figure 3E**), and FAE hydrolyzing activity (**Figure 3F**) were analyzed with increasing concentrations of protein from mouse lung tissue. All protein curves had a high coefficient of determination ($r^2 \geq 0.93$). Increasing levels of isolated protein from mouse lung (1.56–50 μg) for the DGL activity assay (**Figure 3D**) led to associated increases in product recovery (0.001 ± 0.00013–0.034 ± 0.002 nmol/min 19:2 MAG). Mouse lung MAG degradation showed increases in product recovery (0.001 ± 0.000022–0.605 ± 0.02 nmol/min 19:2 FFA) as isolated protein (0.39–50 μg) increased (**Figure 3E**). FAE hydrolyzing activity in mouse lung (**Figure 3F**) also indicated that as protein increased (3.12–50 μg), product recovery also increased (0.003 ± 0.000097–0.022 ± 0.003 nmol/min [$^2H_4$]-PA).

Validation of Enzyme Activity in Jejunum Epithelium

Known inhibitors of associated enzymes were used to validate specificity of each assay. All inhibition curves displayed a high coefficient of determination ($r^2 \geq 0.87$). Activity of DGL in protein isolates from mouse jejunum epithelium (50 μg) was inhibited in a concentration-dependent manner

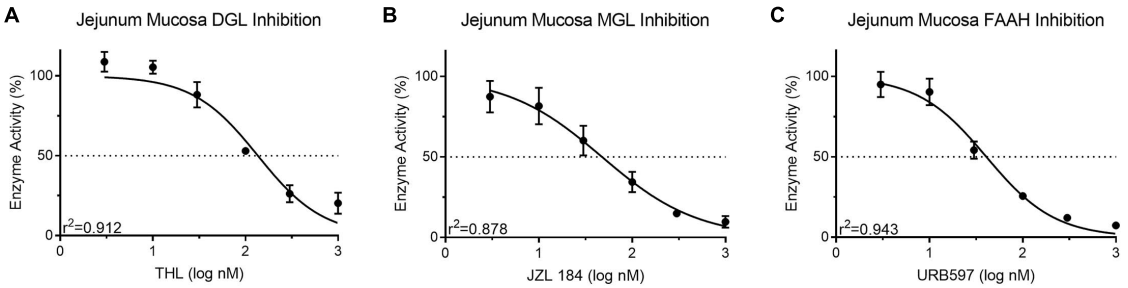

FIGURE 4 | Activity of the MAG biosynthetic enzyme, DGL, in mouse small-intestinal epithelium was dose-dependently inhibited by the DGL inhibitor, THL **(A)** IC_{50} = 136.3 nM. Activity of the MAG degradative enzyme, MGL, in small-intestinal epithelium was dose-dependently inhibited by the MGL inhibitor, JZL 184 **(B)** IC_{50} = 47.62 nM. Activity of the FAE degradative enzyme, FAAH, in small-intestinal epithelium was inhibited by the FAAH inhibitor, URB597 **(C)** IC_{50} = 40.76 nM.

(108.85 ± 6.19–20.39 ± 6.65%; IC_{50} = 133.6 nM) when incubated with the DGL inhibitor, THL (3–1,000 nM) (**Figure 4A**). This IC_{50} for THL was higher than reported (60 nM) for human recombinant DGL (Bisogno et al., 2006). Activity of MGL in protein isolates from mouse jejunum epithelium (10 μg) was inhibited in a concentration-dependent manner (87.42 ± 9.82–9.68 ± 3.59%; IC_{50} = 47.62 nM) when incubated with the MGL inhibitor, JZL 184 (3–1,000 nM) (**Figure 4B**). This IC_{50} for JZL 184 was higher than reported (8 nM) for mouse brain tissue (Long et al., 2009). Activity of FAAH in protein isolates from mouse jejunum epithelium (50 μg) was inhibited in a concentration-dependent manner (94.95 ± 7.89– 7.37 ± 0.001%; IC_{50} = 40.76 nM) when incubated with the FAAH inhibitor, URB597 (3–1,000 nM) (**Figure 4C**). This IC50 for URB597 was higher than reported (5 nM) for rat brain membranes (5 nM) (Piomelli et al., 2006). Collectively, differences in IC50 values for compounds in comparison to other reports suggest possible differential effects due to assay-specific conditions and tissues analyzed.

Validation of Enzyme Activity in Lung

Mouse lung protein isolates (50 μg) displayed a predictable reduction in DAG metabolism when incubated with THL (3–1,000 nM; 90.26 ± 9.07–10.19 ± 0.29%; IC_{50} = 74.64 nM) (**Figure 5A**). This IC_{50} for THL was similar to reported values (60 nM) for human recombinant DGL (Bisogno et al., 2006). Inhibition of MAG metabolism in lung protein isolates (25 μg) with JZL 184 (3–10,000 nM) was incomplete in reducing product recovery (94.99 ± 15.91–49.03 ± 2.32%; IC_{50} = 4,394 nM) (**Figure 5B**). This IC_{50} for JZL 184 was higher than reported (8 nM) for mouse brain tissue (Long et al., 2009). Similarly, incubation of lung protein isolates (25 μg) with ABHD6 inhibitor, WWL 70 (3–10,000 nM), was incomplete in reducing product recovery (87.89 ± 13.69–33.07 ± 1.72%; IC_{50} = 779.9 nM) (**Figure 5C**). This IC_{50} for WWL 70 was also higher than reported (70 nM) for a fibroblast cell line (Li et al., 2007), which again may suggest possible differential effects for compounds due to assay-specific conditions and tissues analyzed. A predictable reduction in MAG metabolism in mouse lung tissue occurred when samples were pre-incubated with 10 μM WWL 70 and JZL 184 (3–10,000 nM; 94.25 ± 6.64–22.32 ± 2.73%; IC_{50} = 514.7 nM) (**Figure 5D**). To further analyze activity of both MGL and ABHD6 in mouse lung tissue, we incubated protein isolates with 10 times the IC_{50} of JZL 184 (43,940 nM) and WWL 70 (7,799 nM) when incubated alone, which significantly reduced MAG metabolism (14.62 ± 3.46%); however, a small amount of residual MAG metabolism persisted under these conditions (**Figure 5E**). Nearly all FAE metabolism in mouse lung protein isolates (10 μg) was inhibited (90.11 ± 8.58–4.15 ± 0.46%; IC_{50} = 22.95 nM) by incubation with URB597 (3–1,000 nM) (**Figure 5F**). This IC_{50} for URB597 was only moderately higher than reported values (5 nM) in rat brain membranes (Piomelli et al., 2006).

Effects of THL Oral Gavage on Levels of MAGs and FAEs in Intestinal Epithelium, Lung, and Circulation

Levels of common MAGs were quantified by UPLC-MS/MS in the jejunum mucosa from vehicle-treated mice that were food deprived for 24 h (**Figure 6A**: 2-AG = 45.81 ± 7.02 nmol/g; 2-DG = 7.53 ± 1.40 nmol/g; 2-PG = 13.52 ± 2.60 nmol/g; 2-OG = 100.71 ± 28.93 nmol/g; 2-LG = 194.08 ± 40.11 nmol/g). Levels of all MAGs were reduced after oral administration of the DGL inhibitor, THL (1 mg), 1 h prior to tissue harvest (2-AG = 8.04 ± 1.52 nmol/g; 2-DG = 0.85 ± 0.13 nmol/g; 2-PG = 4.91 ± 0.51 nmol/g; 2-OG = 5.56 ± 1.10 nmol/g; 2-LG = 24.61 ± 7.48 nmol/g). Vehicle-treated mice displayed no significant changes in levels of MAGs in lung (**Figure 6B**: 2-AG = 4.47 ± 0.44 nmol/g; 2-DG = 1.17 ± 0.07 nmol/g; 2-PG = 3.15 ± 0.26 nmol/g; 2-OG = 1.81 ± 0.13 nmol/g; 2-LG = 0.51 ± 0.10 nmol/g) when compared to mice treated with THL (2-AG = 3.73 ± 0.37 nmol/g; 2-DG = 1.02 ± 0.07 nmol/g; 2-PG = 2.51 ± 0.15 nmol/g; 2-OG = 2.25 ± 0.37 nmol/g; 2-LG = 1.13 ± 0.35 nmol/g). No significant changes in plasma MAGs were observed when comparing THL-treated mice (**Figure 6C**: 2-AG = 29.34 ± 2.18 pmol/mL; 2-DG = 32.82 ± 1.67 pmol/mL; 2-PG 51.99 ± 9.87 pmol/mL; 2-OG = 1.53 ± 0.22 pmol/mL; 2-LG = 105.12 ± 11.41 pmol/mL) with vehicle-treated mice (2-AG = 31.59 ± 3.93 pmol/mL; 2-DG = 44.25 ± 11.63 pmol/mL; 2-PG = 46.63 ± 4.97 pmol/mL; 2-OG = 2.71 ± 0.68 pmol/mL; 2-LG = 156.32 ± 27.64 pmol/mL).

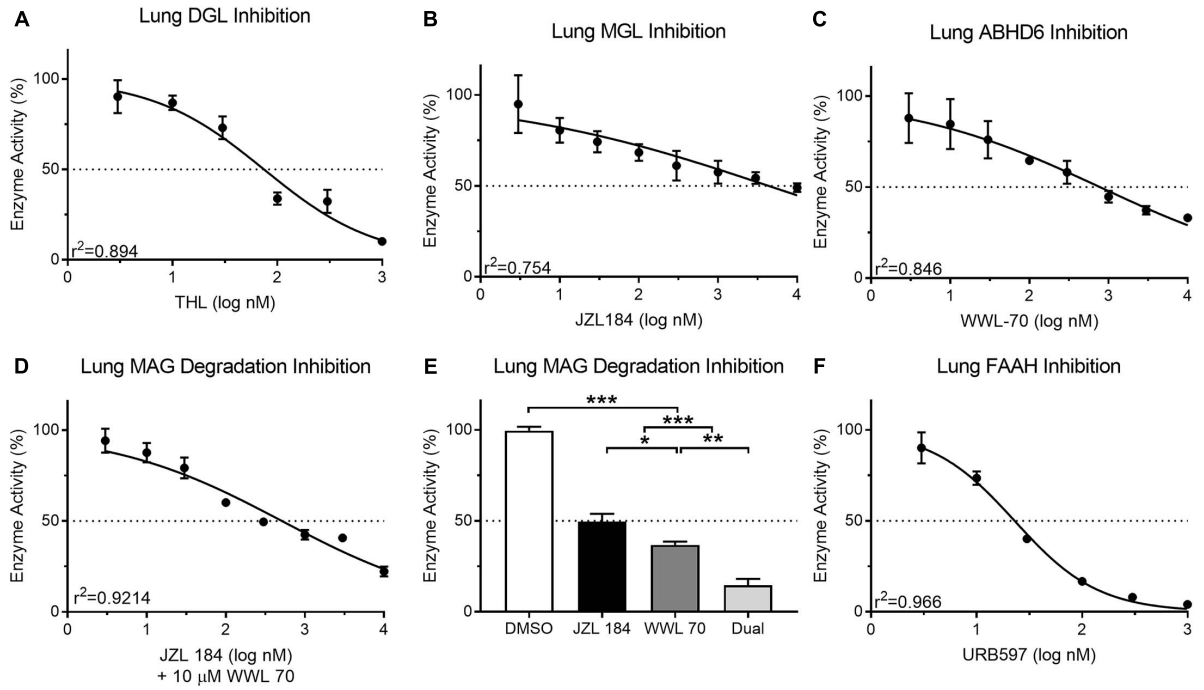

FIGURE 5 | Activity of the MAG biosynthetic enzyme, DGL, in mouse lung protein isolates was dose-dependently inhibited by the DGL inhibitor, THL **(A)** IC_{50} = 74.64 nM. In contrast to small-intestinal epithelium, activity of the MAG degradative enzyme, MGL, in mouse lung was not effectively reduced past 50% with concentrations up to nearly 10 μM of the MGL inhibitor, JZL 184 **(B)** IC_{50} = 4,394 nM. Activity of the MAG degradative enzyme, ABHD6, in mouse lung was inhibited by the ABHD6 inhibitor, WWL 70 **(C)** IC_{50} = 779.9 nM. Dose-dependent inhibition of MAG metabolism in mouse lung tissue was achieved when all samples were also incubated with 10 μM WWL 70 **(D)** IC_{50} = 510.14 nM. When mouse lung was incubated together at 10× the respective IC_{50} for each MAG degradation inhibitor (JZL 184 = 43,940 nM; WWL 70 = 7,799 nM), MAG degradative enzyme activity was reduced to 14.62% **(E)**. Activity of the FAE degradative enzyme, FAAH, in mouse lung protein isolates was inhibited by the FAAH inhibitor, URB597 **(F)** IC_{50} = 22.95 nM.

To assess if THL affected FAE metabolism, levels of commons FAEs were quantified in jejunum mucosa, lungs, and in circulation. Vehicle-treated mice displayed no changes in levels of FAEs in the jejunum mucosa (**Figure 6D**: AEA = 2.85 ± 0.29 pmol/g; OEA = 52.23 ± 6.73 pmol/g; DHEA = 14.53 ± 1.25 pmol/g) when compared to mice that received THL (AEA = 2.41 ± 0.24 pmol/g; OEA = 46.83 ± 9.00 pmol/g; DHEA = 18.97 ± 5.08 pmol/g). Vehicle-treated mice also exhibited no changes in levels of FAEs in lung tissue (AEA = 3.14 ± 0.14 pmol/g; OEA = 92.06 ± 4.99 pmol/g; DHEA = 14.25 ± 2.18 pmol/g) when compared to mice treated with THL (**Figure 6E**: AEA = 2.85 ± 0.08 pmol/g; OEA = 83.75 ± 3.44 pmol/g; DHEA = 16.13 ± 1.19 pmol/g). Plasma concentrations of FAEs were also unaffected when THL-treated mice (**Figure 6F**: AEA = 3.11 ± 0.50 pmol/mL; OEA = 36.12 ± 2.36 pmol/mL; DHEA = 5.62 ± 0.43 pmol/mL) were compared to vehicle-treated mice (AEA = 3.76 ± 0.07 pmol/mL; OEA = 41.19 ± 3.84 pmol/mL; DHEA = 6.41 ± 0.76 pmol/mL). Together, these results suggest that DGL is a primary biosynthetic enzyme in mouse intestinal epithelium. Moreover, these methods can be utilized to manipulate production of MAGs specifically in the intestinal epithelium without affecting activity of DGL in extra-intestinal organs.

DISCUSSION

We describe in this report UPLC-MS/MS-based methods for determining activity of enzymes that control eCB metabolism in distinct mouse mucosal tissues, which can be applied to other tissues of interest. These methods are optimized for quantitating the rate of (i) MAG biosynthesis in intestinal epithelium and lung tissue via DGL, (ii) MAG degradation in intestinal epithelium and lung tissue via MGL, (iii) MAG degradation via ABHD6 in lung tissue, and (iv) FAE degradation in intestinal epithelium and lung tissue via FAAH. Notably, we provide novel methods that do not require radioactive substrates to assess activity of FAAH in mouse mucosal tissues as described elsewhere (Fu et al., 2007; Dainese et al., 2020), and expand and optimize to lung tissue application of our previously-reported UPLC-MS/MS-based assays of DGL and MGL activity in intestinal epithelium (Batugedara et al., 2018; Argueta et al., 2019). Furthermore, the UPLC-MS/MS methods described here are ideal for detecting discrete changes in the activity of enzymes that metabolize eCBs and related lipids given that these enzymatic reactions involve hydrolysis of substrates leading to detectable changes in substrate mass. Moreover, we report significant MAG degradation in mouse lungs via ABHD6, which accounts for up to 66% of metabolism of MAGs in lung tissue when applying the methods provided here. Lastly, we describe an *in vivo* model for intestinal-specific inhibition of

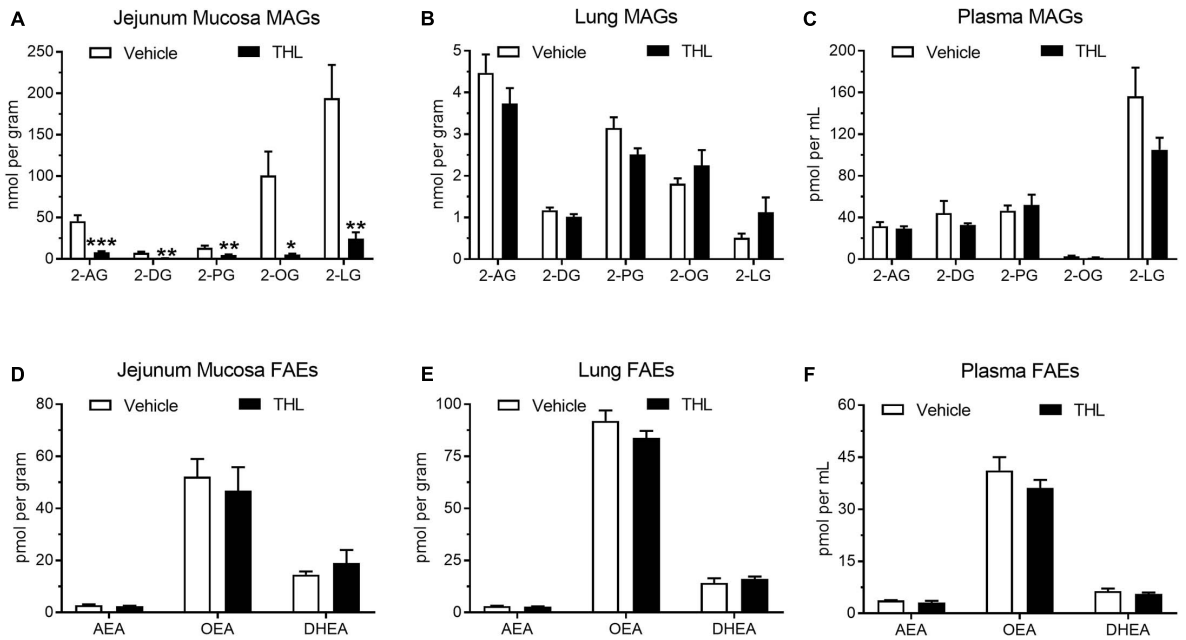

FIGURE 6 | Oral gavage of the DGL inhibitor, THL, inhibited production of MAGs in the upper small-intestinal epithelium **(A)**, but not in lung **(B)** or in circulation **(C)**. THL had no effect on levels of FAEs in upper small-intestinal epithelium **(D)**, lung **(E)**, and circulation **(F)**. *-indicates p-value < 0.05, **indicates p-value < 0.01, ***indicates p-value < 0.001, $n = 6$–8.

MAG production in mice, with results that suggest DGL is a primary biosynthetic enzyme for MAGs in mouse intestinal epithelium. These methods can be applied to studying the activity of eCB system-related enzymes under physiological conditions and changes in their activity associated with pathophysiological conditions (e.g., diet-induced obesity).

Several biochemical and molecular assays are common for analyzing eCB system activity including qPCR-based analysis of expression of genes for specific components of the system (e.g., eCB biosynthetic and degradative enzymes) (Argueta et al., 2019; Avalos et al., 2020). Importantly, however, quantitating levels of gene expression does not provide a full – and at times accurate – depiction of the state of eCB system activity. For example, we reported that mice rendered obese by exposure to a "western-style" diet high in fats and sugars, when compared to lean control mice fed a low-fat/sugar diet, display elevated levels of 2-AG and other MAGs in the small-intestinal epithelium, and this heightened eCB activity at local CB_1Rs promotes overeating associated with diet-induced obesity (Argueta et al., 2019). We then analyzed expression of genes for a host of eCB system components, including DGL and MGL, in order to assess if changes in expression of these key biosynthetic and degradative MAG enzymes, respectively, are responsible for elevated MAG levels. We found that expression of genes for the dominant isoform of DGL in the mouse intestinal epithelium, DGLβ, was *decreased* in obese mice versus lean controls. We then performed an *ex vivo* analysis of activity of DGL using our UPLC-MS/MS-based functional assay described here and found that activity of DGL in tissue from the intestinal epithelium was *increased* in obese mice versus lean controls. This result suggests that despite decreases in expression of genes for DGL, activity of DGL was increased, which provides evidence that elevated levels of 2-AG and other MAGs in the intestinal epithelium of obese mice occur due to increases in their biosynthesis. Therefore, solely analyzing expression of genes for eCB system components inherently does not provide an accurate assessment of activity of the system under physiological and pathophysiological conditions. A combined approach is recommended in order to gain a more comprehensive understanding of eCB system activity.

We report that incubation of protein isolates from the lungs of healthy male mice with an ABHD6 inhibitor (WWL 70) led to a concentration-dependent blockade of MAG degradation by up to 66% of activity, which suggests that ABHD6 is a major enzyme involved in the degradation of MAGs in the murine lung. ABHD6 is well characterized in the mouse brain where it contributes to ∼5–10% of MAG degradation in brain homogenates and ∼50% of MAG degradation in neuronal cultures (Marrs et al., 2010; Savinainen et al., 2012); however, MGL is thought to be the dominant MAG degradative enzyme throughout the periphery. Evidence indicates macrophages produce significant 2-AG in the presence of LPS and WWL 70, which suggests a substantial role for ABHD6 activity in these cells (Bottemanne et al., 2019). Thus, it is plausible that resident lung macrophages may be responsible for ABHD6-mediated MAG metabolism. In addition, inhibition of MGL and ABHD6 in lung tissue decreased total MAG degradation in mouse lung homogenates; however, full inhibition was not achieved. Thus, alternate enzymatic pathways in the mouse lung may contribute in part to degradation of MAGs including ABHD12, which has similar catalytic capabilities as ABHD6 (Fiskerstrand et al., 2010; Savinainen et al., 2012).

Indeed, other studies suggest that mutations in ABHD12 may contribute to neurodegenerative diseases due to alterations in eCB metabolism (Fiskerstrand et al., 2010). It is also plausible that higher concentrations of WWL 70 or JZL 184 may be necessary for full *in vitro* inhibition of MAG metabolism in mouse lung tissue. Moreover, macrophages express the FAE-metabolizing enzyme, NAAA, which in addition to FAAH, may contribute to FAE hydrolysis in lung (Tsuboi et al., 2007). Nonetheless, we report a near full inhibition of mouse lung FAE hydrolysis with URB597; however, our methods and conditions described above for assaying activity of FAAH differed from those reported for assaying activity of NAAA and may contribute to differential effects for inhibitors under assay conditions that favor FAAH over NAAA activity (e.g., differing centrifugation speeds) (Solorzano et al., 2009; Scalvini et al., 2020). It is also notable that the UPLC/MS/MS assays we describe here utilize tissue homogenates that, in contrast to assays using purified enzymes, contain a variety of enzymes in addition to DGL, MGL, and AHD6 that may contribute to hydrolysis of corresponding substrates. This possibility is reflected in the experiments using THL for inhibition of DAG hydrolysis (**Figure 5A**) and JZL 184 and WWL 70 for inhibition of MAG hydrolysis (**Figure 5D**), which identify two inflection points for inhibition of activity. Indeed, THL is not entirely selective for DGL (see Hoover et al., 2008) and at higher concentrations, may be affecting the activity of enzymes other than DGL.

The described methods in this work rely on highly sensitive UPLC-MS/MS technology which provides several advantages in assaying enzyme activity. The inclusion of internal standards and non-endogenous substrates in these assays increases the accuracy of the quantitative results when compared to other assays of enzyme activity, including fluorogenic assays. Indeed, the addition of fluorogenic residues on a substrate, including those previously reported for assaying FAAH activity (Ramarao et al., 2005), may alter its enzymatic conversion, decreasing assay sensitivity (Su et al., 2006; Ohira et al., 2018). Furthermore, isomerases are known to change molecular orientation of the substrate without changing its mass (Lambeth and Julian, 2019); however, changes in molecular orientation may lead to changes in solubility and, ultimately, changes in UPLC-MS/MS retention time. Therefore, the coupling of MS to the chromatographic step of liquid chromatography provides a sensitive method for determining enzyme activity with advantages over other methods described.

We previously reported that a 24-hour fast stimulates production of 2-AG in rat jejunal epithelium, and production of 2-AG under these conditions is blocked following oral gavage with the DGL inhibitor, THL, which suggests that DGL is a primary biosynthetic enzyme for 2-AG in this tissue in rats (DiPatrizio et al., 2015). We now provide evidence that THL blocks production of 2-AG along with several other MAG species in mouse intestinal epithelium, which suggests that DGL is a key enzyme in the biosynthesis of MAGs in the small-intestinal epithelium of rodents. Furthermore, these results suggest that THL does not broadly affect production of eCBs in the intestinal epithelium because levels of anandamide and other FAEs were not affected in this tissue. Moreover, THL had no effect on levels of MAGs in the lung or in circulation, which suggests that THL suspended in PEG was not likely absorbed into circulation and can be utilized via these methods to block production of MAGs selectively in the rodent intestinal epithelium.

Collectively, these functional assays are useful for analyzing tissue-specific activity of eCB biosynthetic and degradative enzymes under physiological and pathophysiological conditions that may be associated with dysregulated eCB metabolism. Furthermore, these methods can be adapted and used as a guide for analyzing activity of eCB biosynthetic and degradative enzymes in other tissues of interest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee University of California, Riverside.

AUTHOR CONTRIBUTIONS

NVD and MBW: concept and design. PAP and CPW: standard curve generation. BA: MGL protein curve generation. MBW: schematic, all other protein curves, inhibition curves, and intestinal/lung lipid content. DA: circulating lipid content. MBW: data analysis and interpretation. NVD and MBW: drafting the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.699712/full#supplementary-material

Supplementary Figure 1 | Representative chromatograms including retention times and predicted masses of products from the reactions of the DGL **(A)**, MGL/ABHD6 **(B)**, and FAAH assays **(C)** and for the internal standards for the DGL **(D)** and MGL/ABHD6/FAAH assays **(E)**.

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The Influence of Supplemental Dietary Linoleic Acid on Skeletal Muscle Contractile Function in a Rodent Model of Barth Syndrome

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Barth syndrome is a rare and incurable X-linked (male-specific) genetic disease that affects the protein tafazzin (Taz). Taz is an important enzyme responsible for synthesizing biologically relevant cardiolipin (for heart and skeletal muscle, cardiolipin rich in linoleic acid), a critical phospholipid of mitochondrial form and function. Mutations to Taz cause dysfunctional mitochondria, resulting in exercise intolerance due to skeletal muscle weakness. To date, there has been limited research on improving skeletal muscle function, with interventions focused on endurance and resistance exercise. Previous cell culture research has shown therapeutic potential for the addition of exogenous linoleic acid in improving Taz-deficient mitochondrial function but has not been examined *in vivo*. The purpose of this study was to examine the influence of supplemental dietary linoleic acid on skeletal muscle function in a rodent model of Barth syndrome, the inducible Taz knockdown (TazKD) mouse. One of the main findings was that TazKD soleus demonstrated an impaired contractile phenotype (slower force development and rates of relaxation) *in vitro* compared to their WT littermates. Interestingly, this impaired contractile phenotype seen *in vitro* did not translate to altered muscle function *in vivo* at the whole-body level. Also, supplemental linoleic acid attenuated, to some degree, *in vitro* impaired contractile phenotype in TazKD soleus, and these findings appear to be partially mediated by improvements in cardiolipin content and resulting mitochondrial supercomplex formation. Future research will further examine alternative mechanisms of dietary supplemental LA on improving skeletal muscle contractile dysfunction in TazKD mice.

Keywords: soleus, contractile kinetics, time to peak twitch, half-relaxation time, cardiolipin, tafazzin

INTRODUCTION

Skeletal muscles play many important roles in the body. As a result, muscle weakness can limit mobility, impair balance, and make breathing difficult, impacting quality of life. Barth syndrome is an example of muscle weakness and, although rare, is a serious genetic disorder that primarily affects males (Barth et al., 1999). Historically, boys with Barth syndrome die

of heart failure or infection. Improved diagnoses and treatment strategies aimed at cardiac and immune dysfunction (Zegallai and Hatch, 2021) have resulted in significantly improved survival rates (Clarke et al., 2013). However, skeletal muscle weakness (Bohnert et al., 2016; Bittel et al., 2018; Hornby et al., 2019) and exercise intolerance (Spencer et al., 2011; Bashir et al., 2017; Hornby et al., 2019) persist and worsen over time, impacting individuals' physical function and independence (Mazar et al., 2019). Interventions directed at skeletal muscle weakness have demonstrated modest improvements in exercise tolerance with endurance exercise (Cade et al., 2016) and muscle strength with resistance exercise (Bittel et al., 2018). However, it is unknown if alternative treatment strategies could also/further improve skeletal muscle function in Barth syndrome.

The primary genetic defect characteristic of Barth syndrome is a mutation to the gene that encodes for tafazzin (Taz; Bione et al., 1996). Taz is a transacylase that remodels cardiolipin (CL; Xu et al., 2006), a phospholipid found predominately in the inner mitochondrial membrane with a fatty acyl chain composition made up of mostly linoleic acid (LA) in heart and skeletal muscle (Schlame, 2013). CL is important for mitochondrial structure (cristae folding, organization of membrane-associated proteins that are part of the electron transport chain), which in turn influences function (energy production; Claypool, 2009). As such, less than optimal functioning of Taz, as seen with Barth syndrome, results in reduced CL content and LA composition, resulting in dysfunctional mitochondria. Thus, treatment strategies aimed at improving CL content rich in LA may prove helpful in improving mitochondrial form and function and, in turn, skeletal muscle function in Barth syndrome patients.

Upon the discovery that impaired CL remodeling was one of the key driving forces behind the pathophysiology of Barth syndrome, it was logical to examine the role of supplemental LA as a potential therapy. To date, three studies have examined the influence of exogenous LA on Barth syndrome, examining cultured fibroblasts (Valianpour et al., 2003), cardiomyocytes (Wang et al., 2014), and lymphoblasts (Xu et al., 2016) from Barth syndrome patients. Incubation of cells with LA led to increases in LA-rich CL (Valianpour et al., 2003; Wang et al., 2014), reduced monolysocardiolipin (MLCL):CL ratio (Wang et al., 2014; Xu et al., 2016), and improved mitochondrial respiration and contractile function (Wang et al., 2014). Despite these positive findings in cell culture, to our knowledge, no studies have examined the influence of supplemental LA on CL content and composition, and skeletal muscle function at the level of the whole organism. The preclinical Taz knockdown (TazKD) mouse model has been extensively used in the literature because it closely recapitulates Barth syndrome pathologies seen in human patients (Ren et al., 2019), including skeletal muscle weakness (Acehan et al., 2011; Soustek et al., 2011) and exercise intolerance (Powers et al., 2013; Schafer et al., 2018; Goncalves et al., 2021). As such, this preclinical model may prove important in testing the possibility and efficacy of supplemental LA treatment.

Past literature has demonstrated that dietary intakes of lipids influence membrane fatty acid composition due to constant membrane remodeling (Ferreri et al., 2016). However, this relationship does not apply to all types of dietary lipids. Rodent skeletal muscle membranes appear relatively unresponsive to dietary saturated and monounsaturated lipids yet somewhat responsive to polyunsaturated fatty acids (PUFA), namely, n-3 PUFA as a percent of total PUFA or "PUFA balance" when fed a moderate dietary intake of lipids (25% of total energy intake) over 8 weeks (Abbott et al., 2010). Specifically, skeletal muscle membranes were very responsive to diets with a low PUFA balance (n-3 PUFAs making up <10% of total dietary PUFAs; slope = 0.98) and somewhat responsive to diets with a moderate to high PUFA balance (n-3 PUFAs making up >30% of total dietary PUFAs; slope = 0.16; Abbott et al., 2010). It is also important to note that the low PUFA balance diets were high in LA (35–70% of total lipids) compared to the moderate to high PUFA balanced diets, where LA was less than 30% of total lipids (Abbott et al., 2010). Although this study examined the response of all membrane phospholipids indiscriminately, prior literature suggests that CL is responsive to changes in dietary lipids in liver (Feillet-Coudray et al., 2014), heart (Feillet-Coudray et al., 2014), and skeletal muscle (Fajardo et al., 2015). Here, we examined the influence of moderate dietary lipid intake (25% of total energy) with high LA (70% of total lipids) and low PUFA balance (<10%) on skeletal muscle CL content and composition, MLCL:CL ratio and skeletal muscle contractile function in a mouse model of Barth syndrome.

MATERIALS AND METHODS

Animals and Diets

As approved by the animal utilization protocol, 16-10-01, a colony of TazKD mice generated from breeders originally purchased from Jackson Laboratories (stock number 014648), were housed at Brock University in the Comparative Biosciences Facility. TazKD was induced *in utero* and maintained post-natal by administering doxycycline (625 mg/kg chow; Envigo TD.01306) as described previously (Acehan et al., 2011). All mice were allowed access to food and water *ad libitum* and were housed in an environmentally controlled room with a standard 12:12-h light–dark cycle. Mice offspring positive for the Taz small hairpin RNA transgene were identified by PCR using primers (forward: 5'-CCATGGAA TTCGAACGCTGACGTC-3'; reverse: 5'-TATGGGCTAT GAACTAATGACCC-3'). Non-transgenic littermates treated with the doxycycline containing diet were used as WT controls. Upon weaning, TazKD and WT littermates were fed a normal (CON) or high LA safflower oil (25% of total energy; Envigo TD.180388) diet (**Table 1**), both containing doxycycline, for a period of 8 weeks (TazKD CON, TazKD LA, WT CON, and WT LA). Over the course of the 8-week dietary treatment, mice were weighed every other day. Following the 8-week dietary intervention, all mice underwent an *in vivo* measurement to assess grip strength (Bioseb, Chaville, France; Mandillo et al., 2008). 24 h following the *in vivo* measurement, mice were euthanized *via* cervical dislocation while under isoflurane anesthetic. Soleus was extracted and either snap frozen in liquid nitrogen and stored at −80°C (lipid analyses,

TABLE 1 | Nutrient content of the diets.

Ingredient	Chow diet	LA diet
Protein (% kcal)	24	18
Carbohydrate (% kcal)	58	57
Sucrose	10	10
Lipid (% kcal)		
Soybean oil	18	–
Safflower oil	–	23
Flaxseed oil	–	2
Vitamin mix (g/kg)[1]	10	10
Mineral mix (g/kg)[2]	35	35

[1]*Vitamin mix contained A (15 IU/g), D_3 (1.5 IU/g), E (110 IU/kg), K_3 (50 mg/kg), thiamin (17 mg/kg), riboflavin (15 mg/kg), niacin (10 mg/kg), B_6 (18 mg/kg), pantothenic acid (33 mg/kg), B_{12} (0.08 mg/kg), biotin (0.4 mg/kg), folate (4 mg/kg), and choline (1,200 mg/kg)*
[2]*Mineral mix contained calcium (1%), phosphorus (0.7%), sodium (0.2%), potassium (0.6%), chloride (0.4%), magnesium (0.2%), zinc (70 mg/kg), manganese (100 mg/kg), copper (15 mg/kg), iodine (6 mg/kg), iron (200 mg/kg), and selenium (0.23 mg/kg).*

qPCR, and Western blotting) or processed immediately (*in vitro* muscle contraction). In a separate group of animals, following the same 8-week dietary intervention, animals were euthanized *via* cervical dislocation while under isoflurane anesthetic, and whole hindlimb tissue was extracted for subsarcolemmal mitochondria isolation. All animal procedures were approved by the Animal Care and Utilization Committee at Brock University (file #18-03-01) in compliance with the Canadian Council of Animal Care.

In vitro Muscle Contractile Function

Soleus from one hindlimb was dissected from distal to proximal tendon after being secured with braided silk sutures and was immediately mounted in an *in vitro* skeletal muscle testing system (Model 1200a; Aurora Scientific Inc.) for contractile measures as previously reported (Gittings et al., 2012; Mikhaeil et al., 2017). Data acquisition was performed using the Model 600a software, version 1.60 (Aurora Scientific Inc.) and muscle stimulation *via* flanking platinum electrodes driven by a Model 701B bi-phase stimulator (Aurora Scientific Inc.). Muscles were suspended in a jacketed organ bath-containing oxygenated (95% O_2 and 5% CO_2) Tyrode's solution (121 mm NaCl, 5 mm KCl, 24 mm $NaHCO_3$, 0.4 mm NaH_2PO_4, 0.5 mm $MgCl_2$, 1.8 mm $CaCl_2$, 5.5 mm D-glucose, and 0.1 mm EDTA, pH 7.4) maintained at 25°C (Lannergren et al., 2000), and left to equilibrate. To determine the optimal length (Lo) for maximal twitch force, muscles resting at just-taut length were stimulated every ~10 s as muscle length was increased in small increments until peak active force was observed. Muscle length at Lo was determined using digital Vernier calipers. After preliminary procedures, the maximum isometric twitch and tetanic forces were obtained at Lo. Twitch force (P_t) was measured as the maximum force produced in response to a single stimulation (i.e., 1 Hz, 0.1 msec pulse width; **Figure 1**), whereas peak tetanic force (P_o) was determined by brief (500 msec) supramaximal stimulation frequency (100 Hz). Time to peak tension (TPT, the elapsed time from the onset to the peak of isometric force development) and half-relaxation time (½RT, elapsed time from the peak to 50% of isometric force development) of twitch force records were calculated. Rates of force development ($+dP/dt$) and relaxation ($-dP/dt$) were calculated as the maximal slope of the rise and fall in force during a twitch, respectively. Isolated muscles were also subjected to force-frequency (1–100 Hz) and fatigue (70 Hz for 350 ms every 2 s for 5 min) protocols (Fajardo et al., 2016). Following the collection of contractile data, muscles were removed from the bath and the sutures were cut off immediately distal to the muscle–tendon junctions. Muscles were briefly blotted dry to remove excess liquid, and mass was determined on a standard mass balance. The calculation for physiological cross-sectional area was done as previously reported, using the optimal length and mass of each muscle (Grange et al., 2002).

qPCR

RNA was isolated from frozen muscle using a Qiagen RNeasy Plus Kit (Qiagen, 74,136). cDNA was generated using EcoDry RNA to cDNA double-primed Reverse Transcriptase kit (Clontech, #639549). qPCR assays were performed on an ABI StepOnePlus Real-Time PCR System instrument (Applied Biosystems, #4376592) with KAPA SYBR FAST (Sigma, #KM4103) master mix and amplification efficiency-optimized primers (Integrated DNA Technologies, Coralville, IA). The primers used included Taz (forward 5' CCC TCC ATG TGA AGT GGC CAT TCC 3'; reverse 5' TGG TGG TTG GAG ACG GTG ATA AGG 3'; Acehan et al., 2011) and β-actin (forward 5' AAG AGC TAT GAG CTG CCT GA 3'; reverse 5' ACG GAT GTC AAC GTC ACA CT 3'; Huang et al., 2017). Threshold cycle (Ct) values were recorded and analyzed using the ΔΔCt method with expression of β-actin used as a reference gene.

Lipid Analyses

Total lipids from muscle homogenates (1.25 mg) were extracted (Folch et al., 1957), and CL content was analyzed by high-performance thin-layer chromatography (HPTLC) (Fajardo et al., 2017). MLCL and CL were separated by HPTCL on a separate plate (Lopalco et al., 2017), and MLCL:CL ratio was subsequently calculated using densitometry (ImageJ, Ver 1.53 k, NIH, United States). LA composition of CL was analyzed by gas chromatography (Bradley et al., 2008; Fajardo et al., 2017).

Western Blotting

Western blotting was performed to examine the protein expression of Taz as previously described (Fajardo et al., 2017) with the following modifications. Proteins from muscle homogenates (2 μg) were solubilized using Laemmli buffer (Laemmli, 1970) and then electrophoretically separated using 12% standard glycine-based SDS-PAGE. Separated proteins were then transferred onto 0.2 μm nitrocellulose membranes (Immuno-Blot, BioRad Inc., CA, United States) using a semi-dry transfer setting on the Trans-Blot Turbo Transfer System (BioRad Inc.). Membranes were immunoprobed with primary antibodies directed against Taz (Lu et al., 2016) diluted in 5% (w/v) milk in tris-buffered saline tween, then immunoprobed with a goat-anti mouse horseradish peroxidase conjugated secondary antibody (Jackson Immuno Research Labs, West Grove, PA), and detected

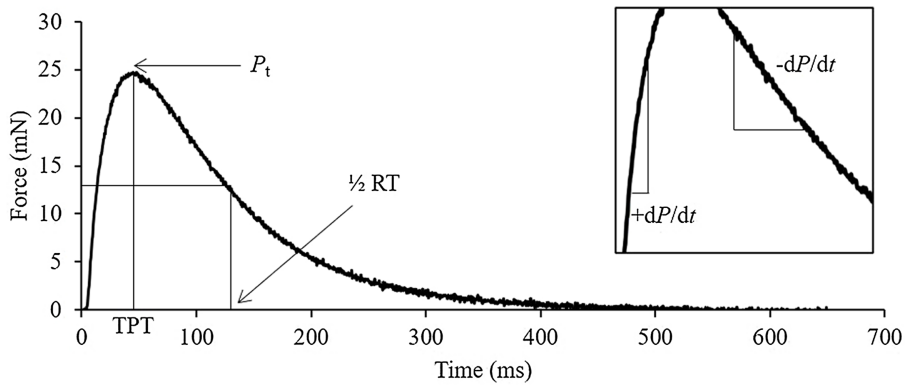

FIGURE 1 | Representative soleus twitch trace identifying the key quantifiable measures of contraction. P_t, peak twitch; TPT, time to peak tension; ½ RT, half-relaxation time; +dP/dt, rate of force development; and −dP/dt, rate of force relaxation.

with Clarity Western ECL Substrate with a BioRad Chemi Doc Imager (BioRad Inc.).

Mitochondrial Supercomplex Analyses

Subsarcolemmal mitochondria were isolated from fresh whole hindlimb tissue as previously reported (Stefanyk et al., 2010). Blue-native polyacrylamide gel-electrophoresis was used to assess mitochondrial supercomplex formation as previously reported (Jha et al., 2016). Densitometry of supercomplexes from membranes was quantified (ImageJ, Ver 1.53 k, NIH, United States) and expressed in reference to WT Con.

Statistics

All values are expressed as the mean ± standard error (SE). All statistical analyses were performed using SPSS Statistics for Windows, version 25 (SPSS Inc., Chicago, Ill., United States). Two-way ANOVAs (genotype and diet) were performed on all data sets followed by a Tukey's post-hoc analysis. For force-frequency curves, force was normalized to maximal force and a three-way mixed plot ANOVA was used to examine the main effects of frequency, genotype, diet, and their potential interactions. Frequency at 50% maximal force (F_{50}) was calculated using the Hill equation. For fatigue curves, area-under-the-curve values were obtained for each muscle then averaged before conducting statistical comparisons. A value of $p < 0.05$ was considered significant for all tests.

RESULTS

Taz Transcription and Translation

The presence of doxycycline in the diet (625 mg/kg) for 8 weeks significantly reduced Taz mRNA content in soleus of TazKD mice to ~10% of WT littermates (**Figure 2A**). This resulted in an absence of Taz protein in soleus of TazKD mice compared to WT littermates (**Figure 2B**). The presence of supplemental LA had no effect on Taz transcription and translation.

Animal Morphometrics and *in vivo* Muscle Function

TazKD mice were slower growing compared to WT littermates, regardless of diet (**Figure 3A**). This resulted in a significantly lower final body weight (**Figure 3B**). However, soleus weight as a fraction of body weight was not significantly different between genotypes or diet (**Figure 3C**). At the end of the dietary treatment, there was no difference between genotypes or diet in combined forelimb-hindlimb grip strength when controlled for body weight (**Figure 3D**).

Cardiolipin Content and Composition and MLCL:CL Ratio

CL content was significantly lower in TazKD soleus compared to WT, which was somewhat attenuated with supplemental LA in that TazKD was no longer significantly different from WT (**Figure 4A**). CL 18:2n6 composition was also lower in TazKD soleus compared to WT but was not affected by supplemental dietary LA (**Figure 4B**). MLCL:CL ratio was higher in TazKD soleus compared to WT and did not appear to be influenced by supplemental dietary LA (**Figure 4C**).

In vitro Soleus Contractile Function

Soleus from TazKD mice have impaired contractile properties compared to WT (**Figure 5A**), as demonstrated by a significantly slower TPT (**Figure 5B**) and ½ RT (**Figure 5C**). The addition of supplemental dietary LA significantly reversed the slow TPT in TazKD mice to levels similar to WT littermates. In terms of ½ RT, this was somewhat attenuated in TazKD with dietary supplemental LA but did not reach significance. Similar to previous research, absolute force production at higher frequencies was lower in TazKD compared to WT littermates but this was not impacted by dietary supplemental LA (**Figure 6A**) nor was it different between genotypes and diet when expressed as a percent of max force (**Figure 6B**) or the frequency at 50% of maximal force (**Figure 6C**). When examining peak twitch force, peak tetanic force, rates of force production, rates of force relaxation (**Table 2**), and rates of fatiguability (**Figure 7**),

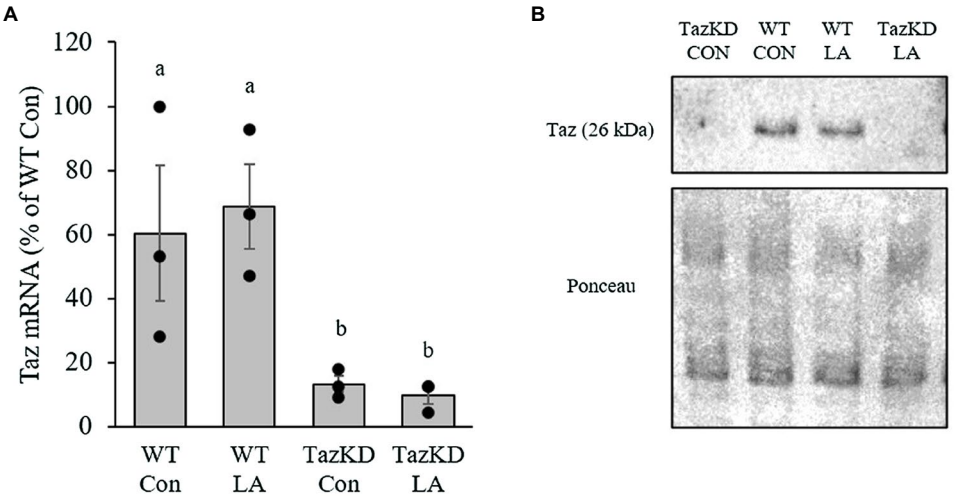

FIGURE 2 | Administration of doxycycline in either control or supplemental linoleic acid diet resulted in a significant reduction in tafazzin (Taz; **A**) mRNA and **(B)** protein content in soleus of Taz knockdown (TazKD) male mice compared to their wild-type littermates. Values are means ± SEM, $n = 3$ for Taz mRNA. WT, wild type; TazKD, Taz knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

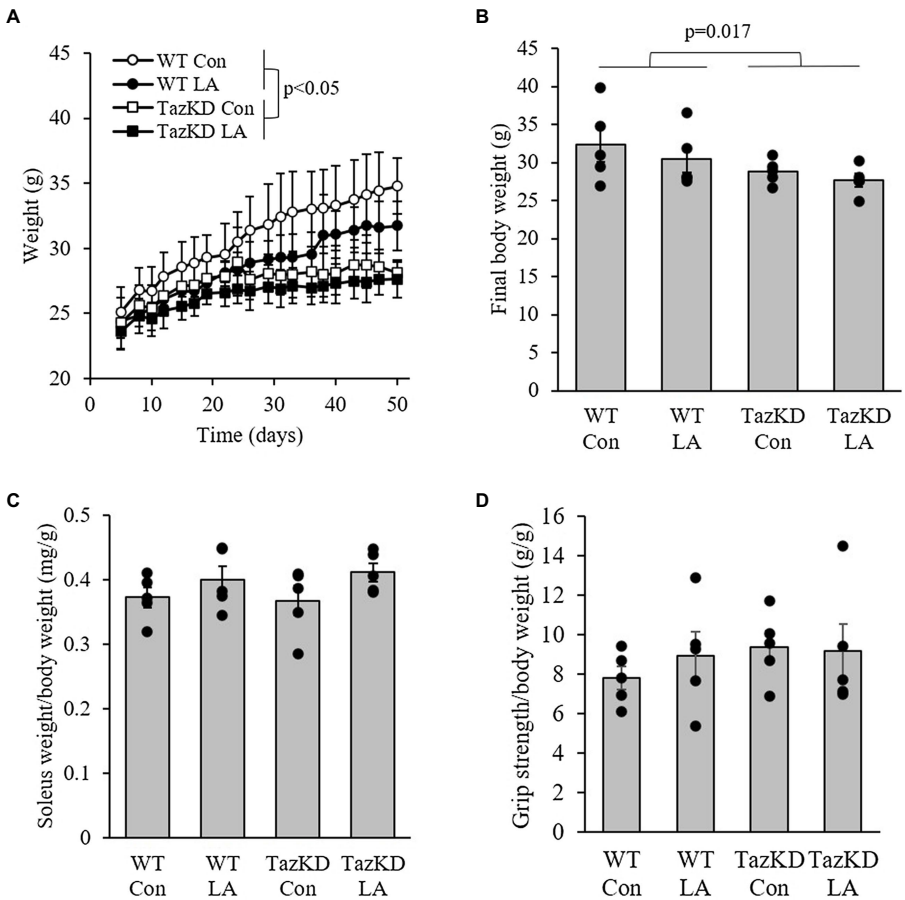

FIGURE 3 | TazKD male mice were **(A)** slower growing and **(B)** had lower final body weight compared to their wild-type counterparts regardless of diet over the 8-week intervention. **(C)** Soleus weight as a fraction of body weight and **(D)** *in vivo* grip strength were not different between genotypes and diet. Values are means ± SEM, $n = 6$. WT, wild type; TazKD, Taz knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

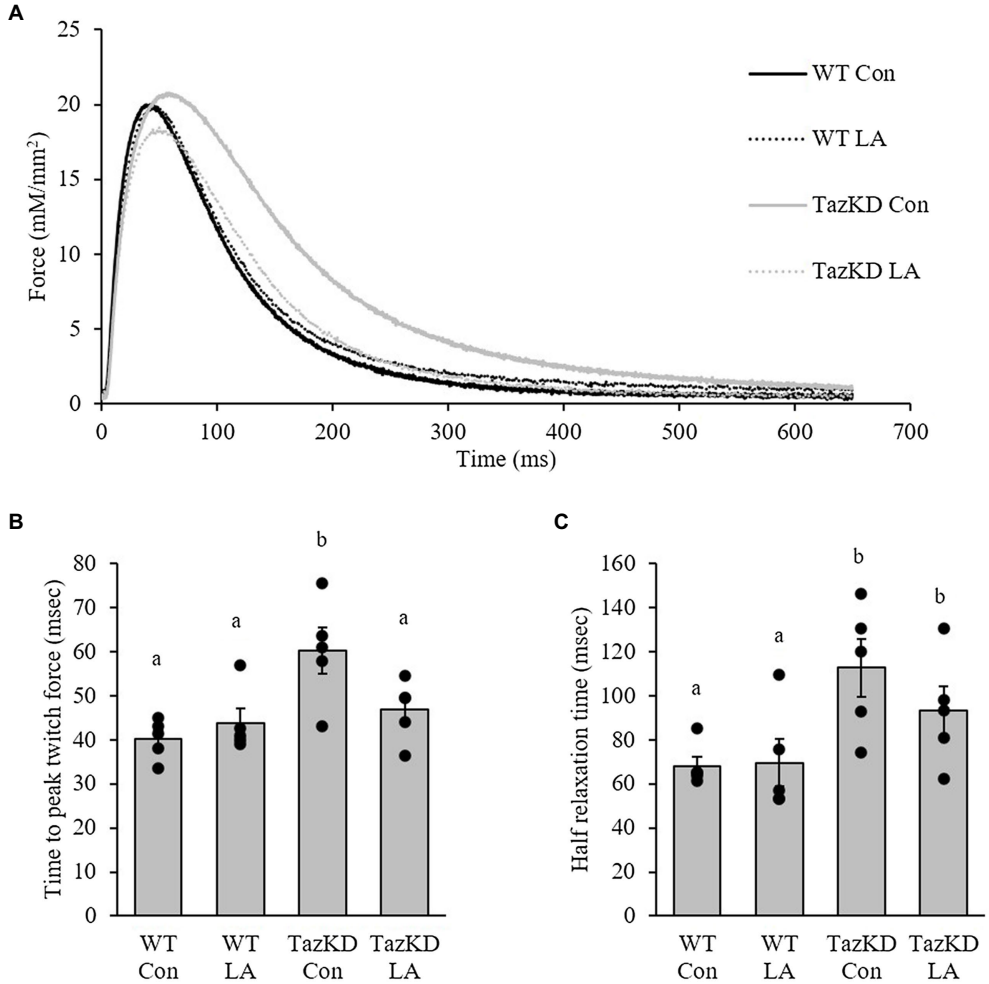

FIGURE 4 | 8 weeks of supplemental linoleic acid diet attenuated, but not significantly, the doxycycline-induced alterations in **(A)** cardiolipin content but had no influence on **(B)** cardiolipin linoleic acid content or **(C)** MLCL:CL ratio in soleus of TazKD male mice compared to their wild-type littermates. Values are means ± SEM, $n = 2$–6; no statistical difference between values with the same letter ($p < 0.05$). CL, cardiolipin; WT, wild type; TazKD, Taz knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

only rates of force relaxation showed a genotype-specific difference.

Mitochondrial Supercomplex Formation

Subsarcolemmal mitochondria isolated from whole hindlimb from TazKD mice had lower supercomplex formation compared to WT littermates (**Figure 8**). Although 8 weeks of dietary supplemental LA somewhat attenuated the impaired supercomplex formation, the finding was not significant ($p = 0.14$).

DISCUSSION

To our knowledge, this is the first study to fully characterize an impaired contractile phenotype in the pre-clinical rodent model of Barth syndrome and the potential efficacy of dietary supplemental LA to reverse this phenotype. Similar to previous studies, administration of doxycycline to TazKD mice *in utero* and post-weaning resulted in decreased skeletal muscle Taz mRNA, protein, CL content, CL 18:2n6 composition, and increased MLCL:CL ratio (Acehan et al., 2011; Soustek et al., 2011; Ikon et al., 2018; Johnson et al., 2018; Goncalves et al., 2021), along with reduced weight gain (Acehan et al., 2011; Cole et al., 2016; Ikon et al., 2018; Johnson et al., 2018; Goncalves et al., 2021). The main findings are (1) TazKD soleus demonstrated slower force development and relaxation *in vitro* as key contributors to this impaired contractile phenotype, (2) the impaired contractile phenotype seen *in vitro* in TazKD compared to WT did not translate to altered muscle function *in vivo*, and (3) supplemental LA attenuated, to some degree, soleus *in vitro* impaired contractile phenotype in TazKD mice, which appears to not be fully mediated by CL content and composition.

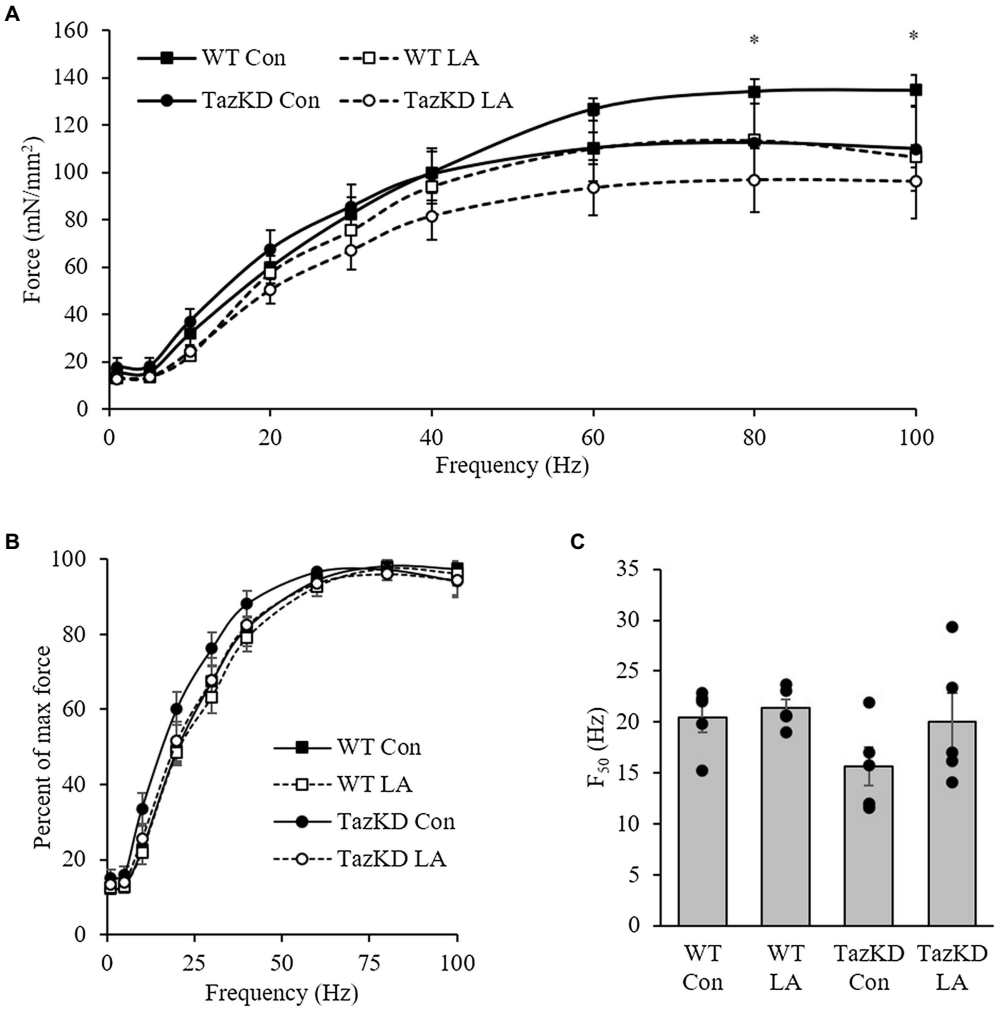

FIGURE 5 | TazKD compared to wild-type male mice fed control diet show altered twitch kinetics as demonstrated by **(A)** the representative trace. **(B)** Time to peak twitch force was slower in TazKD soleus compared to WT and was attenuated by the LA diet. In comparison, **(C)** half-relaxation time was also slower in TazKD soleus compared to WT and was somewhat attenuated with the LA diet. Values are means ± SEM, $n = 5$; no statistical difference between values with the same letter ($p < 0.05$). WT, wild type; TazKD, Taz knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

TazKD Soleus Demonstrated Slower Force Development and Relaxation *in vitro* as Key Contributors to an Impaired Contractile Phenotype

This study represents the first to demonstrate altered skeletal muscle contractile kinetics *in vitro* in a mouse model of Barth syndrome. Soleus from TazKD male mice had slower TPT, ½ relaxation time (½RT), and rate of force relaxation ($-dP/dt$). Changes in skeletal muscle contractile kinetics can be influenced by muscle fiber-type composition. Soleus in mice is ~31% type I, 49% type IIA, 12% type IIX, and 3% type IIB (Augusto et al., 2004; Bloemberg and Quadrilatero, 2012) and demonstrates fiber-type shifting when challenged by unloading (Fajardo et al., 2017), endurance exercise (Kruger et al., 2013), lithium supplementation (Whitley et al., 2020), and aging (Bott et al., 2017) to name a few. To date, no research has examined the fiber-type composition of TazKD skeletal muscle. It is not likely that the observed changes in TPT, ½ RT, and $-dP/dt$ in the current study would be due to changes in fiber-type composition as there were no differences in peak twitch force, peak tetanic force, or resistance to fatigue, skeletal muscle contractile variables that correlate with fiber-type composition (Stephenson et al., 1998). It is more likely that given Taz is an important enzyme to remodel CL necessary for proper mitochondrial function (Paradies et al., 2019), slower force development and relaxation in TazKD soleus may be related to the energetics of muscle contraction and relaxation, respectively.

One possible contributor to the slowed contractile kinetic phenotype seen in the current study is ATP availability. ATP, supplied by mitochondrial oxidative phosphorylation, is essential to fuel myosin ATPase for cross bridge cycling of myosin and actin during contraction and calcium reuptake into the sarcoplasmic reticulum by the sarco(endo)plasmic reticulum calcium ATPase (SERCA) during relaxation. Impaired

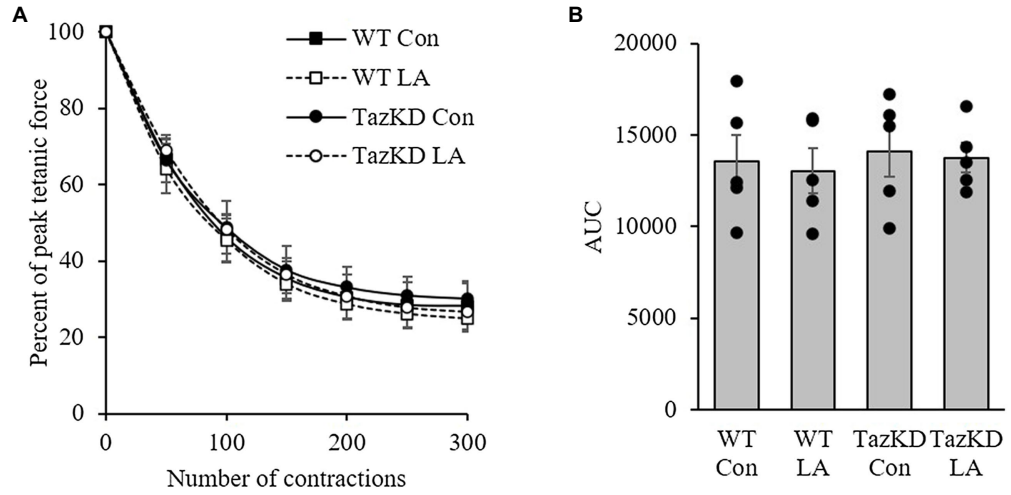

FIGURE 6 | 8 weeks of supplemental linoleic acid diet did not influence soleus fatigability expressed as **(A)** percent peak tetanic force and **(B)** area under the curve in TazKD male mice, or their wild-type littermates compared to the control chow diet. Values are means ± SEM, $n = 5$. WT, wild type; TazKD, tafazzin knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

TABLE 2 | Isometric muscle function in response to 8 weeks of control or supplemental linoleic acid diet in soleus of doxycycline-induced tafazzin knockdown mice and their wild-type littermates.

	WT		TazKD	
	Con	**LA**	**Con**	**LA**
P_t (mN)	20 ± 2	20 ± 4	21 ± 5	18 ± 2
P_t/CSA (mN/mm^2)	15 ± 2	14 ± 2	18 ± 5	14 ± 2
P_o (mN)	168 ± 26	154 ± 14	132 ± 16	138 ± 14
P_o/CSA (mN/mm^2)	127 ± 23	110 ± 15	116 ± 19	106 ± 13
+dP/dt (mN/msec)	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
−dP/dt (mN/msec)*	0.17 ± 0.02	0.15 ± 0.03	0.10 ± 0.04	0.13 ± 0.03
P_t:P_o	0.12 ± 0.01	0.13 ± 0.02	0.15 ± 0.02	0.13 ± 0.02

*Values are means ± SEM, n = 5; WT, wild-type; TazKD, tafazzin knockdown; Con, chow diet; LA, linoleic acid-supplemented diet; P_t, peak twitch force; CSA, cross-sectional area; P_o, peak tetanic force; +dP/dt, rate of force development; and −dP/dt, rate of force relaxation. *Denotes main effect for genotype.*

mitochondrial supercomplex formation in TazKD compared to WT seen in the current study may limit ATP supply as there is a clear link between mitochondrial structure, specifically supercomplex formation, and mitochondrial bioenergetics (Baker et al., 2019). As such, the reduced CL remodeling due to the knockdown of Taz influences mitochondrial form which may result in impaired mitochondrial bioenergetics, altering muscle contractile kinetics. In fact, individuals with progressive external ophthalmoplegia, a form of mitochondrial myopathy with reduced ATP production, demonstrate slowed contraction and relaxation kinetics in tibialis anterior muscle compared to healthy controls (Moglia et al., 1995). Given the reduced mitochondrial supercomplex formation seen in the current study combined with the characteristics of impaired mitochondrial bioenergetics seen with Barth syndrome, including the TazKD rodent model (Ghosh et al., 2019; Ren et al., 2019), it stands to reason that this could be a significant contributor to the slowed contractile kinetic phenotype. Future research will examine possible correlations between mitochondrial bioenergetics and the impaired contractile kinetic phenotype in TazKD mice.

An alternative hypothesis that may contribute to the slowing of contractile kinetics in TazKD soleus may be impaired calcium homeostasis. To facilitate the contraction-relaxation cycle of skeletal muscle, calcium moves out and in of the sarcoplasmic reticulum, respectively. To regulate mitochondrial ATP production to match skeletal muscle contractile ATP demands, the increased cytosolic calcium seen during contraction enters mitochondria through the mitochondrial calcium uniporter (MCU) and, in turn, upregulates mitochondrial-dependent ATP supply (Finkel et al., 2015). Previous research has shown that cellular models of Barth syndrome (Taz knockout C_2C_{12} myoblasts and Barth syndrome patient-derived lymphocytes) have reduced levels of MCU and that CL is required for proper MCU activity (Ghosh et al., 2020). It is plausible that the reduced CL content in TazKD impaired soleus mitochondrial MCU content and function, preventing a match between ATP supply and demand, resulting in slower contraction and should be the focus of the future research.

An important contributor to potential impaired calcium homeostasis, and resulting slow contractile kinetics, specifically ½ RT and −dP/dt, in TazKD soleus is SERCA. SERCA transports calcium back into the SR post-contraction, and its function is reflected in its activity (total amount of calcium moved into the SR) and efficiency (ATP cost per calcium ion moved; Periasamy and Kalyanasundaram, 2007). Changes to SERCA function could delay calcium uptake and, in turn, muscle relaxation. There are seven known isoforms of SERCA with two found in adult skeletal muscle, SERCA1a in fast twitch fibers and SERCA2a in slow twitch

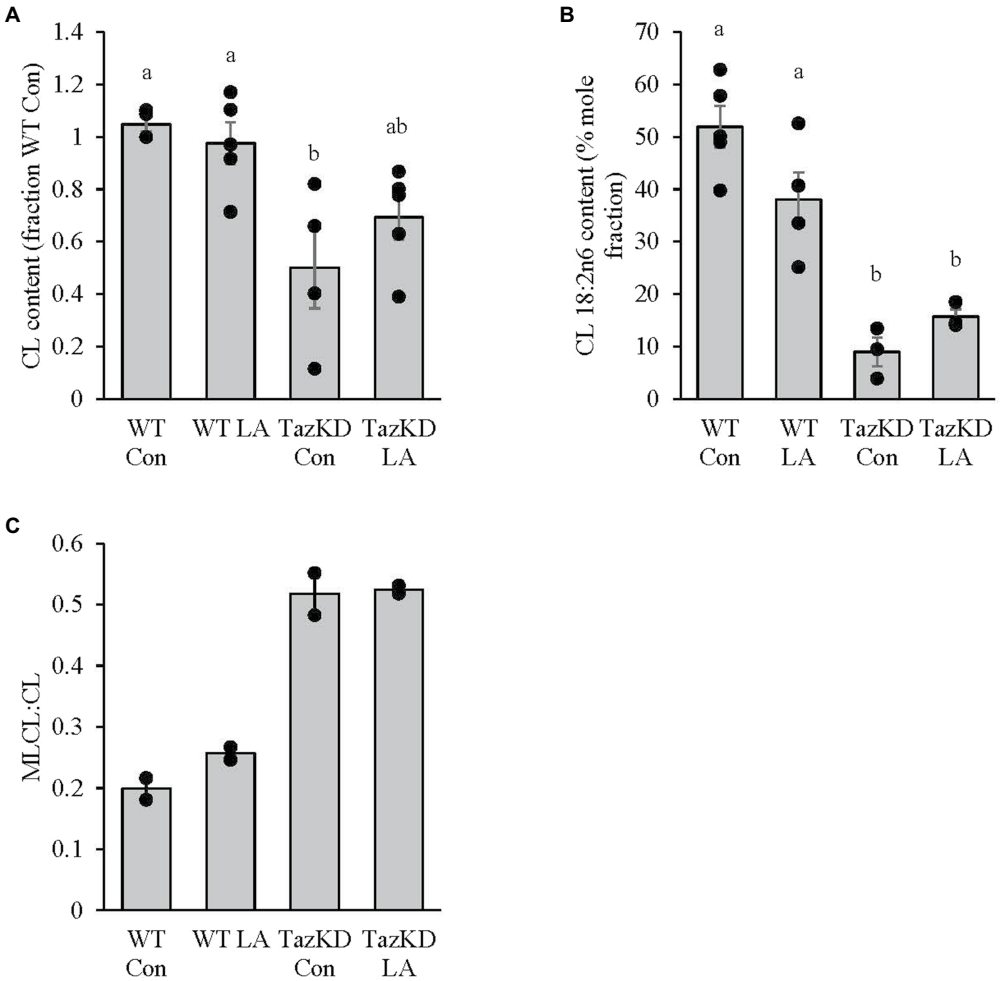

FIGURE 7 | TazKD soleus demonstrated **(A)** lower absolute twitch force production at higher frequencies compared to WT but no significant differences between genotypes and diet when expressed as **(B)** a percent of max force or **(C)** the frequency at 50% of maximal force (F_{50}). Values are means ± SEM, $n = 3$–5. WT, wild type; TazKD, Taz knockdown; Con, chow diet; and LA, linoleic acid-supplemented diet.

fibers and cardiac tissue (Periasamy and Kalyanasundaram, 2007). Previous research has shown that disruptions to SERCA2a gene expression prolonged soleus relaxation time (Sjaland et al., 2011). In addition, left ventricle of TazKD mice compared to wild type demonstrated no difference in SERCA2a expression but had lower SERCA activity that correlated with greater SERCA2a tyrosine nitration, a marker of oxidative/nitrosative stress (Braun et al., 2019). Thus, changes to SERCA2a function in TazKD soleus, be it nitrosative stress or protein expression, may be a contributing factor to slower relaxation kinetics. Future research should examine SERCA2a protein expression and tyrosine nitration in TazKD soleus.

The force-frequency relationship in soleus of TazKD mice compared to wild-type littermates demonstrated a lower force generated at 80 and 100 Hz, which is in agreement with a previous study that showed impaired soleus force production at 100 and 160 Hz in 2-month old mice (Soustek et al., 2011). The impaired force in TazKD soleus did not translate into a significant reduction in the frequency required for 50% of maximal force (F_{50}). However, it is important to note that in addition to impaired soleus relaxation time highlighted above, knocking out SERCA2a resulted in a 9% reduction in F_{50} (Sjaland et al., 2011). This, once again, points to a potential role of SERCA2a form and function in TazKD contractile kinetics that warrants further investigation.

The Impaired Contractile Phenotype Seen *in vitro* in TazKD Compared to WT Did Not Translate to Altered Muscle Function *in vivo*

Slowed contractile kinetics in TazKD soleus compared to WT did not translate to changes in grip strength. A lack of change to *in vivo* muscle function is similar to previous

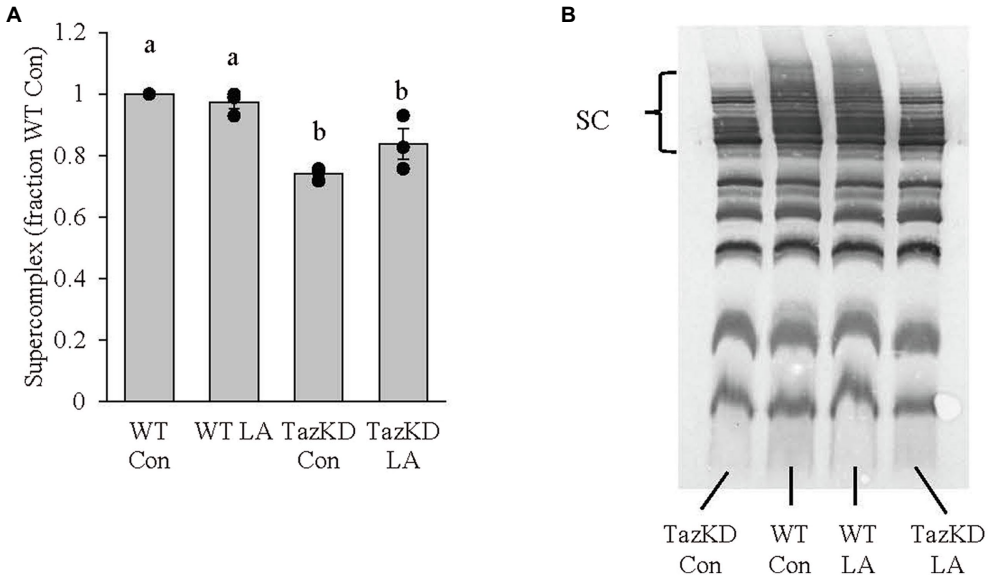

FIGURE 8 | Subsarcolemmal mitochondria isolated from whole hindlimb of TazKD male mice demonstrated **(A)** lower supercomplex formation compared to wild-type littermates, which was not significantly increased ($p = 0.14$) with 8 weeks of supplemental linoleic acid diet. **(B)** Example membrane from blue-native polyacrylamide gel electrophoresis. Values are means ± SEM, $n = 3$. WT, wild type; TazKD, tafazzin knockdown; Con, chow diet; LA, linoleic acid-supplemented diet; and SC, supercomplex.

research in that locomotor activity in TazKD mice did not differ from WT (Cole et al., 2018; Goncalves et al., 2021). Grip strength is a common non-invasive *in vivo* evaluation of muscle force (Ge et al., 2016; Martinez-Huenchullan et al., 2017). However, despite slower rates of contraction and relaxation in soleus, there were no changes in peak twitch force. In addition, contractile properties of one hindlimb muscle may not be representative of all limb muscles. Skeletal muscles vary in terms of fiber-type composition and, in turn, contractile properties (Schiaffino and Reggiani, 2011). It is currently unknown if the doxycycline-induced knockdown of Taz in other muscles with similar oxidative capacities as soleus, such as red gastrocnemius and vastus intermedius (Bloemberg and Quadrilatero, 2012), would show similar impaired contractile kinetics. Future research should examine whole hindlimb contractile properties (Gerlinger-Romero et al., 2019) in TazKD mice to determine if the impaired contractile kinetics is expressed at the level of the whole hindlimb.

Supplemental LA Attenuated, to Some Degree, the *in vitro* Impaired Contractile Phenotype in the Soleus of TazKD Mice

Diet supplemented with high LA safflower oil for 8 weeks somewhat attenuated, although not statistically significant, the total amount of CL and mitochondrial supercomplex formation, but did not increase CL LA content or reduce the MLCL:CL ratio in TazKD soleus. These findings are somewhat in contrast to previous research where supplemental LA reduced the MLCL:CL ratio in Barth syndrome patient-derived lymphoblast (Xu et al., 2016) and cardiomyocytes (Wang et al., 2014), increased CL content and CL 18:2n6 composition in Barth syndrome patient-derived fibroblasts (Valianpour et al., 2003), and increased cardiac tetralinoleoyl CL (TLCL) in rodent models of heart failure (Chicco et al., 2008; Mulligan et al., 2012; Maekawa et al., 2019) that have impaired CL biosynthetic pathways (Saini-Chohan et al., 009). It is plausible that dietary supplemental LA may have stimulated mitochondrial biogenesis (discussed more below) resulting in a non-significant increase in total CL content in TazKD soleus. However, in the face of reduced taz, this limited CL remodeling and, in turn, CL 18:2n6 composition. Future research should examine all CL species in TazKD soleus using mass spectrometry technology and their relative response to high dietary LA.

The current study demonstrates that time to peak twitch and, to a lesser degree, half-relaxation time were improved with the high LA safflower oil diet with non-statistically significant increases in CL content, CL LA composition, and mitochondrial supercomplex formation. It may be argued that dietary LA imposed, in part, some biologically significant influence on improving the impaired contractile kinetic phenotype of TazKD soleus by way of improved mitochondrial form (CL content and composition and supercomplex formation) and, in turn, mitochondrial bioenergetic function. However, these findings may not fully address the LA supplemented diet-mediated improved contractile kinetic phenotype in TazKD soleus. A complementary hypothesis may be related to the ability of dietary lipids to influence skeletal muscle metabolism and energy production needed during contraction and relaxation

independent of changes to mitochondrial structure. It has been reported that the skeletal muscle of Barth syndrome patients (Cade et al., 2019) and TazKD mice (Powers et al., 2013; Goncalves et al., 2021) demonstrate impaired fat oxidation and reliance on glycolytic pathways that produce lactate that is believed to contribute to exercise intolerance. It has also been shown in TazKD mice that bezafibrate, a pan-PPAR agonist, improved cardiac function by increasing mitochondrial biogenesis (Huang et al., 2017; Schafer et al., 2018) with no improvement in TLCL (Huang et al., 2017), and when combined with endurance training, improves exercise tolerance (Schafer et al., 2018). In skeletal muscle, PPARβ/δ is the dominant isoform and activation by lipophilic ligands, including fatty acids, results in increased mitochondrial biogenesis and β-oxidation (Manickam and Wahli, 2017). It can be hypothesized that increased availability of LA may have increased mitochondrial biogenesis and/or β-oxidative capacity in soleus of TazKD mice through PPARβ/δ activation contributing to an improvement to the impaired contractile kinetic phenotype. Future research should examine the role of PPAR as it relates to LA diet-mediated improvement to muscle contraction in TazKD mice.

Summary and Conclusion

This was the first study to demonstrate an impaired contractile phenotype *in vitro* in soleus of the rodent model of Barth syndrome. However, when this model was presented with supplemental LA in the diet, the improvement in the impaired contractile kinetics appears to be somewhat mediated by mitochondrial structural changes due to non-statistically significant increases in CL content and mitochondrial supercomplex formation post-dietary treatment. A complementary hypothesis is that the improved *in vitro* contractile kinetics may also be through a PPAR pathway, potentially improving lipid metabolism to provide energy for contraction and relaxation. Future research should further examine alternative mechanisms of dietary supplemental LA on improving skeletal muscle contractile dysfunction in TazKD mice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Utilization Committee at Brock University.

AUTHOR CONTRIBUTIONS

ME performed the *in vivo* experiments, analyzed the data, and edited the manuscript. DV, MA, MF, and RM assisted in data collection and analyses, and edited the manuscript. SC provided reagents, guidance, and edited the manuscript. PL developed the overall study, collected and analyzed the data, and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Sterols, Oxysterols, and Accessible Cholesterol: Signalling for Homeostasis, in Immunity and During Development

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In this article we discuss the concept of accessible plasma membrane cholesterol and its involvement as a signalling molecule. Changes in plasma membrane accessible cholesterol, although only being minor in the context of total cholesterol plasma membrane cholesterol and total cell cholesterol, are a key regulator of overall cellular cholesterol homeostasis by the SREBP pathway. Accessible cholesterol also provides the second messenger between patched 1 and smoothened in the hedgehog signalling pathway important during development, and its depletion may provide a mechanism of resistance to microbial pathogens including SARS-CoV-2. We revise the hypothesis that oxysterols are a signalling form of cholesterol, in this instance as a rapidly acting and paracrine version of accessible cholesterol.

Keywords: 25-hydroxycholesterol, SREBP pathway, INSIG, HMG-CoA reductase, SARS-CoV-2, hedgehog signalling pathway, cholesterol dependent cytolysin

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INTRODUCTION

Cholesterol is the dominant sterol in animal cells. It is present at a level of about 10–20 fmol/cell (Radhakrishnan et al., 2008; Infante and Radhakrishnan, 2017) and can be found in the membrane of every organelle, with about 60–90% of cellular cholesterol present in the plasma membrane (Lange et al., 1989), where its concentration may be 45 mole % of total lipids (Lange et al., 1989; Das et al., 2013, 2014). The unit mole % of total lipids is used to convey the % contribution in moles of a defined lipid, i.e., cholesterol, to the total lipid content of a membrane or cell. Intracellularly, cholesterol is trafficked between organelles *via* vesicular transport or non-vesicular transport mechanisms involving membrane contact sites (Sandhu et al., 2018; Hoglinger et al., 2019; Naito et al., 2019; Ferrari et al., 2020; Trinh et al., 2020). It may be imported to cells *via* lipoprotein up-take or it can be synthesised *de novo*. Almost every vertebrate cell has the machinery to synthesise cholesterol (Nes, 2011; Cerqueira et al., 2016), and most if not all have the capacity to metabolise it, the first step of which is oxidation to an oxysterol (Schroepfer, 2000). Many invertebrates, including insects and nematodes, are sterol auxotrophs, relying on sterols consumption as part of the diet (Carvalho et al., 2010). Oxysterols are oxidised forms of cholesterol and can cross membranes far quicker than cholesterol (Lange et al., 1995; Meaney et al., 2002). Different cells and tissues generate different oxysterols and like cholesterol can be esterified and transported in the circulation with lipoproteins (Dzeletovic et al., 1995). A small fraction of cholesterol is converted to steroid hormones, but the majority is converted to

bile acids (Russell, 2003; Griffiths and Wang, 2020). Bile acid biosynthesis proceeds predominantly in the liver but minor pathways may be initiated extrahepatically (Babiker et al., 1999; Meaney et al., 2007; Ogundare et al., 2010; Griffiths and Wang, 2020). Bile acids have many functions, they provide an elimination form of cholesterol; in the biliary tract they solubilise and transport cholesterol; and in the small intestine they solubilise dietary lipids and act as antimicrobials. Remarkably, 90% of bile acids entering the small intestine are reabsorbed, and recycled to the liver *via* the enterohepatic system (Hofmann and Hagey, 2014). While oxysterols and bile acids are *bona fide* signalling molecules (Evans and Mangelsdorf, 2014), so is cholesterol in its "accessible" form (Radhakrishnan et al., 2020).

CHOLESTEROL BIOSYNTHESIS, UPTAKE, GENES, ENZYMES, AND REGULATION

Cholesterol Biosynthesis

Cholesterol is synthesised from acetyl-CoA in a pathway involving at least 20 enzymes (**Figure 1**; Goldstein et al., 2006; Nes, 2011; Mazein et al., 2013; Cerqueira et al., 2016). There are five key intermediates in this pathway (i) 6-carbon mevalonate generated by reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA reductase, this represents the rate determining step of the pathway, (ii) 15-carbon farnesyl pyrophosphate (farnesyl-PP), this provides a branch point to nonsteroidal isoprenoids, e.g., geranylgeraniol, dolichols, and ubiquinone, (iii) 30-carbon squalene generated from two farnesyl-PP substrates, the precursor of (iv) squalene-2,3S-epoxide, and (v) 30-carbon lanosterol, the first sterol, formed by the cyclisation of squalene-2,3S-epoxide. Each of the genes coding each of the enzymes in the cholesterol biosynthesis pathway has a sterol regulatory (or response) element (SRE) in its promotor, and is activated by the nuclear form of the master transcription factor sterol regulatory element-binding protein-2 (SREBP-2; Horton et al., 2002, 2003; Goldstein et al., 2006; Mazein et al., 2013; Brown et al., 2018). The low-density lipoprotein (LDL) receptor gene also has a SRE and is regulated by SREBP-2.

SREBP-2 Pathway

High levels of cellular cholesterol lead to elevated cholesterol levels in the endoplasmic reticulum (i.e., >5 mole % endoplasmic reticulum lipids; Radhakrishnan et al., 2008; Das et al., 2014; Infante and Radhakrishnan, 2017) and to a process termed convergent inhibition whereby cholesterol synthesis and expression of the LDL-receptor are reduced restoring cholesterol to its optimal level (Horton et al., 2002; Goldstein et al., 2006; Brown et al., 2018). SREBP-2 is synthesised in the endoplasmic reticulum and immediately binds to the transport protein SCAP (SREBP cleavage-activating protein) which, in the absence of elevated levels of cholesterol transports SREBP-2 to the Golgi where it undergoes two cleavage reactions catalysed by the serine protease S1P (site 1 protease) and the metalloprotease S2P (site 2 protease) to release the active transcription factor that translocate to the nucleus and activates target gene expression (**Figure 2**, upper panel). Mammalian cells produced three

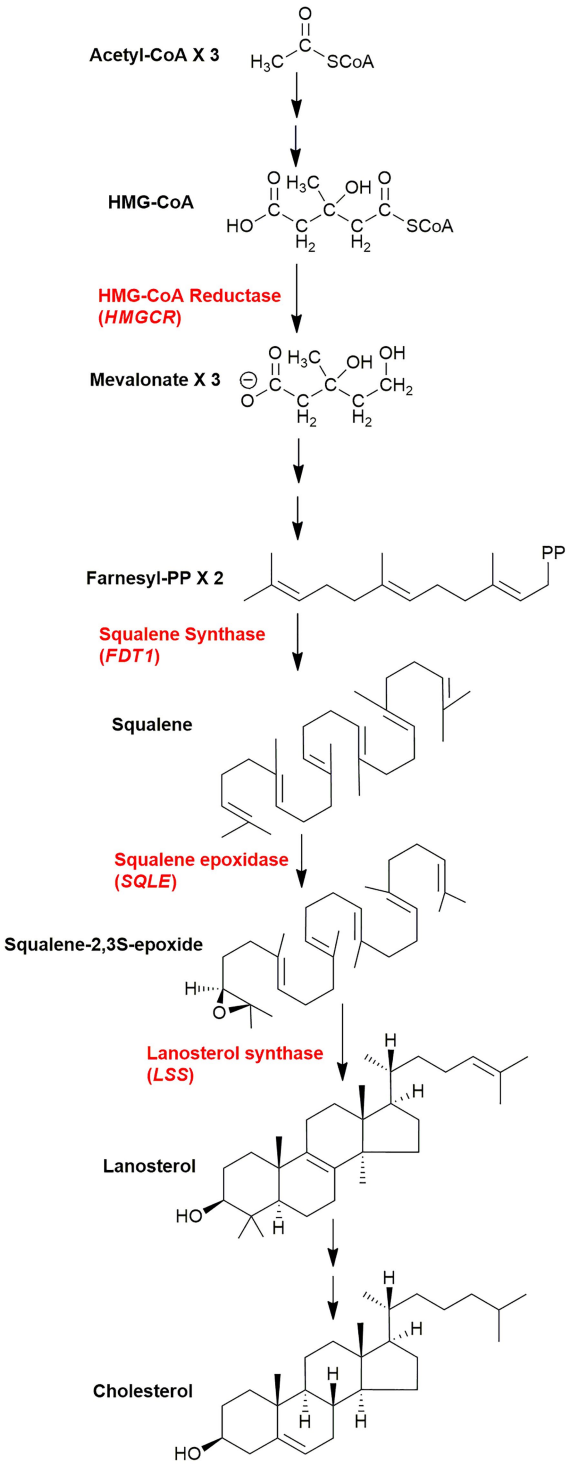

FIGURE 1 | Simplified scheme of the cholesterol biosynthetic pathway. Key enzymes are in red, genes in parenthesis. The pathway post squalene can be found in detail in **Figure 6**. The entire pathway including all steps and enzymes can be found in Mazein et al. (2013).

SREBP isoforms SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are produced by the same gene by the use of different promotors and alternative splicing. In the liver the dominant SREBPs are SREBP-1c and SREBP-2, where SREBP-2 is primarily involved in stimulating cholesterol synthesis while SREBP-1c primarily stimulates fatty acid synthesis (Horton et al., 2002). SREBP-2 also stimulates expression of the LDL-receptor and the endoplasmic reticulum resident protein INSIG (insulin induced gene; Goldstein et al., 2006). When cholesterol levels are elevated in the endoplasmic reticulum [>5 mole % endoplasmic reticulum lipids (Radhakrishnan et al., 2008; Das et al., 2014; Infante and Radhakrishnan, 2017)], cholesterol binds to SCAP which in turn binds to INSIG tethering the SCAP-SREBP-2 complex in the endoplasmic reticulum and preventing transport to the Golgi, and thus SREBP-2 proteolysis and activation (**Figure 2**, central panel). In this way cholesterol regulates its own biosynthesis and uptake by the LDL-receptor (**Figure 2**).

LDL-Receptor, Cholesterol Uptake, and Intracellular Transport

Cells expressing the LDL-receptor take-up cholesterol by a process called receptor mediated endocytosis (Brown and Goldstein, 1986), where LDL particles bind to the LDL receptor located on the plasma membrane and become internalised in endosomes and degraded in the lysosome. Cholesterol esters are hydrolysed by lysosomal acid lipase (*LIPA*; Goldstein et al., 1975; Brown et al., 1976) and non-esterified cholesterol transferred to the lysosomal membrane and ultimately out of the lysosome by the combined action of Niemann-Pick proteins NPC2 and NPC1 (**Figure 3**; Kwon et al., 2009; Li et al., 2016; Infante and Radhakrishnan, 2017). In this way a deficiency in endoplasmic reticulum cholesterol is ultimately restored.

However, there is a conundrum, most of the cell's cholesterol is found in the plasma membrane, while the proteins SREBP-2, SCAP and INSIG are in the endoplasmic reticulum. What's more cholesterol released from the lysosome is believed to travel to the plasma membrane prior to the endoplasmic reticulum (Das et al., 2014; Infante and Radhakrishnan, 2017; Trinh et al., 2020). So how can plasma membrane levels of cholesterol be sensed by proteins located in the endoplasmic reticulum? The answer lies in the concept of "accessible" plasma membrane cholesterol (Das et al., 2014). Three pools of plasma membrane cholesterol have been suggested. A pool of *cholesterol sequestered by sphingomyelin* (SM) comprising about 15 mole % of plasma membrane lipids, a second pool *sequestered in some other way* comprising about 12 mole % of plasma membrane lipids and essential to maintain membrane morphology, and

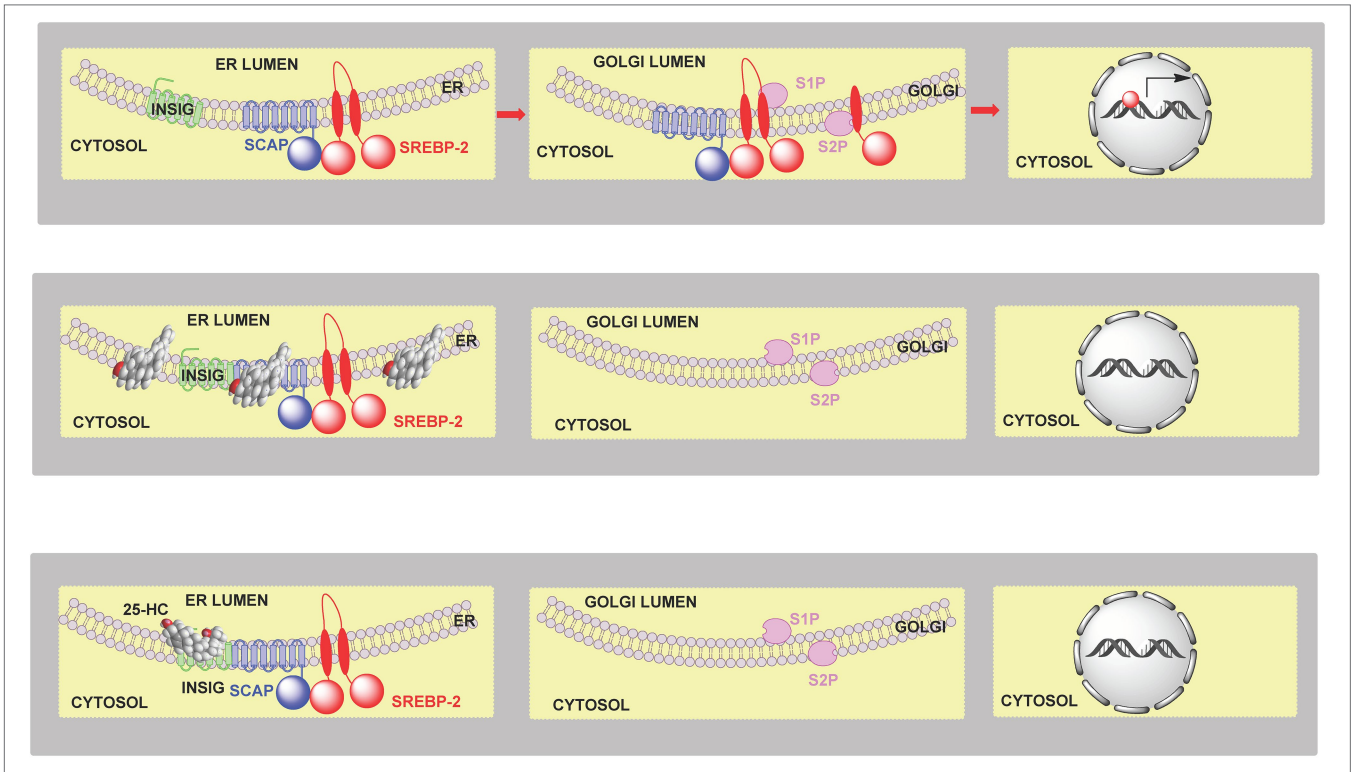

FIGURE 2 | Cartoon representation of the regulation of cholesterol biosynthesis by SREBP-2. **Upper panel**, left to right, SCAP transports SREBP-2 from the endoplasmic reticulum to the Golgi, it is then processed to the active nuclear form which translocates to the nucleus and activates target gene transcription. **Central panel**, when cholesterol levels are elevated (>5 mole % endoplasmic reticulum lipids), cholesterol binds to SCAP, which then binds to INSIG retaining the SCAP-SREBP-2 complex in the endoplasmic reticulum preventing transport to, and formation of the active transcription factor, in the Golgi. **Lower panel**, 25-HC can bind to INSIG tethering the SCAP-SREBP-2 complex in the endoplasmic reticulum and restraining SREBP-2 transport and processing. SREBP-2 target genes include *ACAT2*, *HMGCS*, *HMGCR*, *MVK*, *PMVK*, *MVD*, *IDI1*, *GGPS1*, *FDPS*, *FDT1*, *SQLE*, *LSS*, *CYP51A1*, *TM7SF2*, *MSMO1 (SC4MOL)*, *NSDHL*, *HSD17B7*, *EBP*, *SC5D*, *DHCR7*, *DHCR24*, *LDLR*, *INSIG1*, and *STARD4* (Horton et al., 2002, 2003).

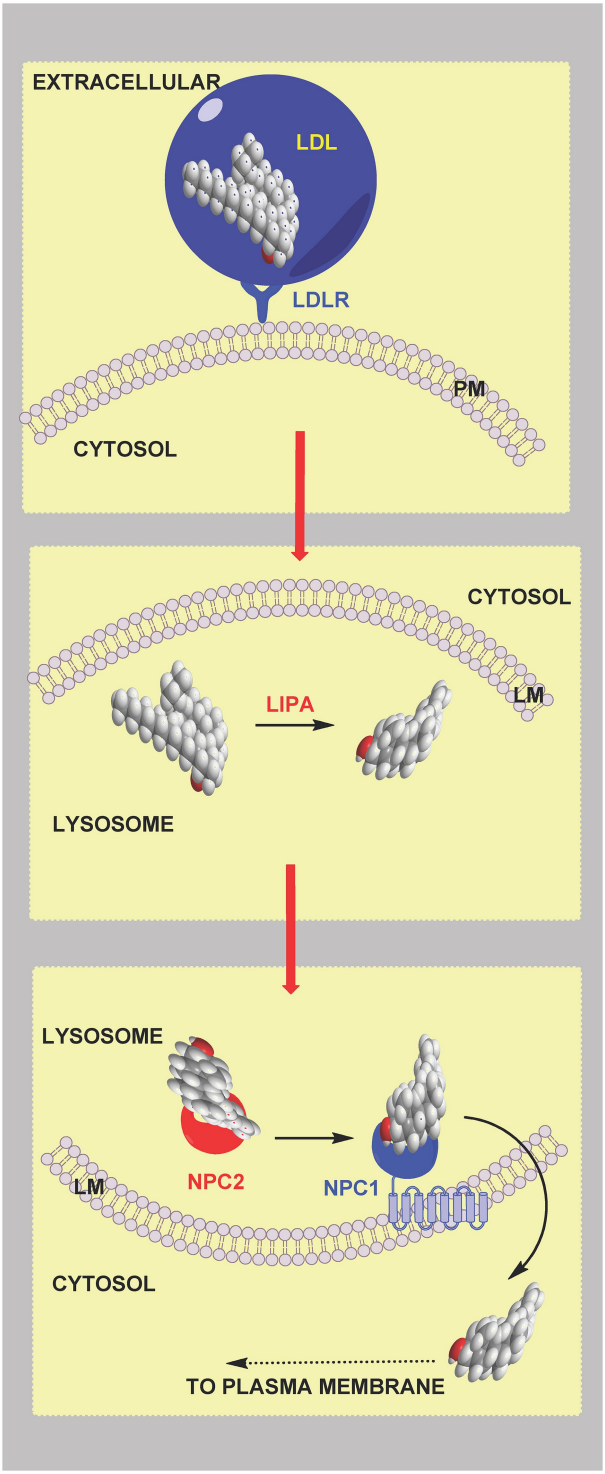

FIGURE 3 | Cartoon representation of cholesterol uptake and transport from the lysosome. LDL particles bind to the LDL-receptor (LDLR) on the extracellular surface of the plasma membrane and are taken up by receptor mediated endocytosis. Endosomes combine with the lysosome where cholesterol esters are hydrolysed by LIPA. Non-esterified cholesterols is transported by soluble NPC2 and membrane bound NPC1 to the lysosomal membrane for export. Exported cholesterol is thought to travel first to the plasma membrane before reaching the endoplasmic reticulum.

an *accessible pool of cholesterol* comprising about 16 mole % of plasma membrane lipids in cholesterol replete cells that signals to the regulatory machinery of the endoplasmic reticulum (Das et al., 2014). The concept of accessible cholesterol is derived from "thermodynamic activity" of species in a condensed phase, where activity, or chemical potential, is dependent on environment. It appears that small changes in the level of accessible cholesterol which are insufficient to cause a measurable change in the total membrane cholesterol are sufficient to be sensed by the SREBP-machinery and regulate cholesterol homeostasis (Das et al., 2014; Infante and Radhakrishnan, 2017). Recent studies indicate that the level of the accessible cholesterol pool dictates the formation of membrane contact sites between the plasma membrane and endoplasmic reticulum through the family of sterol transport proteins called Asters coded by *GRAMD1* genes (Sandhu et al., 2018; Naito et al., 2019; Ferrari et al., 2020; Trinh et al., 2020; Xiao et al., 2021). Aster proteins have (i) a GRAM domain that binds to the plasma membrane in a manner dependent on accessible cholesterol and also glycerolphosphoserine (PS), (ii) a central fold (ASTER domain) resembling the sterol binding fold in StARD (steroidogenic acute regulatory) proteins and (iii) a C-terminal transmembrane domain (Sandhu et al., 2018; Naito et al., 2019; Ferrari et al., 2020; Trinh et al., 2020). When the accessible pool of cholesterol expands, e.g., following cholesterol uptake by the LDL-receptor and transport through the lysosomal system, there is a change in plasma membrane presentation of PS resulting in Aster binding through the GRAM domain and cholesterol transport *via* the central ASTER domain to the endoplasmic reticulum (**Figure 4**). In this way small changes in total plasma membrane and cellular cholesterol are detected by the endoplasmic reticulum sterol regulatory machinery which then provides a mechanism for restoration of the disturbance, rapidly protecting the cell from cholesterol overload.

High Density Lipoprotein Particles and Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is the mechanism by which cholesterol is returned to the liver from peripheral tissues *via* high density lipoprotein (HDL) particles. Non-esterified cholesterol is transported across the plasma membrane to apolipoprotein A1 (ApoA1) or pre-β HDL by ATP-binding cassette transporters ABCA1 and ABCG1, two translation products of liver X receptor (LXR) target genes (see section Liver X Receptors), expression of which is activated by oxysterol binding to heterodimers of LXR and retinoid X receptor (RXR; Venkateswaran et al., 2000; Evans and Mangelsdorf, 2014; Wang and Tontonoz, 2018). Free cholesterol then becomes esterified in HDL particles by lecithin cholesterol acyl transferase (LCAT) with the acyl donor derived from position 2 of a glycerophosphocholine. LXR target genes also include apolipoproteins and proteins involved in lipoprotein remodelling further demonstrating a link between LXRs and RCT (Wang and Tontonoz, 2018). Significantly, mutations in *ABCA1* can lead to Tangier disease, an inborn error of metabolism characterised by reduced plasma HDL cholesterol and accumulation of cholesterol in peripheral tissue (Rust et al., 1999).

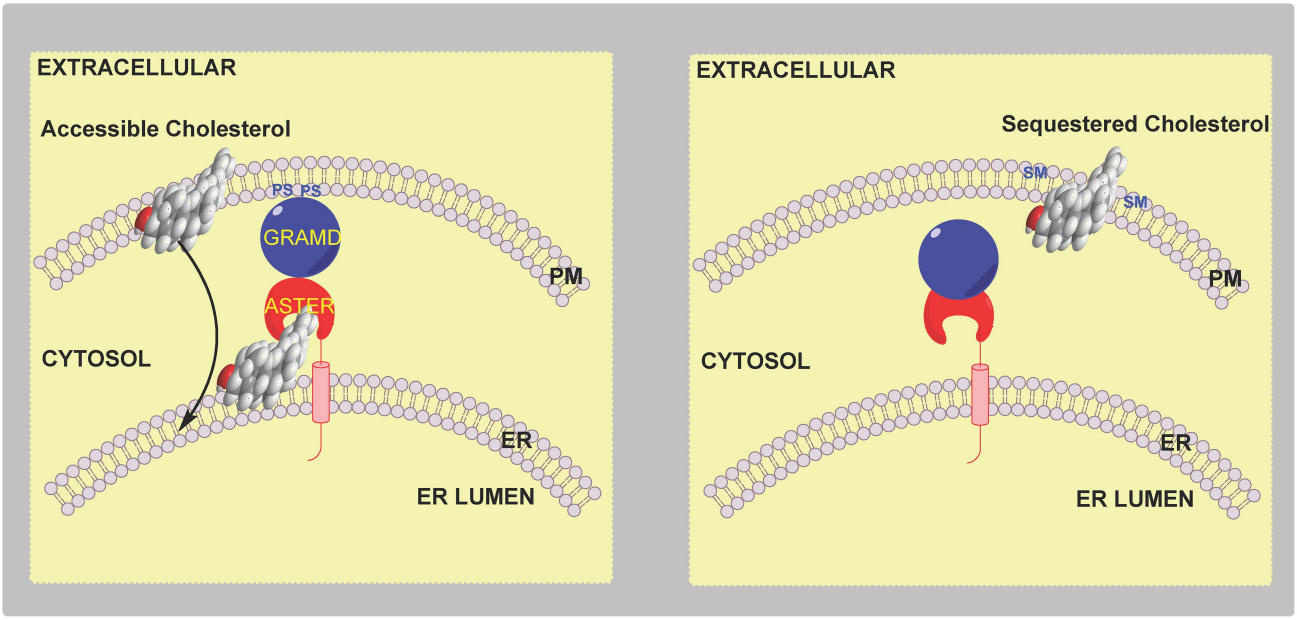

FIGURE 4 | Cartoon illustration of the involvement of Aster proteins in cholesterol transport and regulation. **Left panel**, an elevation in accessible cholesterol leads to presentation of PS on the inner leaflet of the plasma membrane and formation of a membrane contact site *via* the GRAM domain of the endoplasmic reticulum resident protein Aster. Accessible cholesterol is then transferred by the ASTER domain of the protein from the plasma membrane to the endoplasmic reticulum to be sensed by the SREBP-machinery. **Right panel**, when accessible cholesterol in the plasma membrane is below a critical level there is no binding of the Aster protein to the plasma membrane.

Scavenger receptor class B member 1 (SR-B1) acts as the major receptor for HDL cholesterol (Acton et al., 1996). It is abundant in the liver and steroidogenic tissue, and facilitates the selective uptake of cholesterol from HDL (Glass et al., 1983). In the liver surplus cholesterol is converted into bile acids or excreted into bile. Unlike the situation with the LDL-receptor, little is known about the pathway taken by HDL-derived cholesterol beyond SR-B1 mediated uptake. However, Aster proteins may again be the link between accessible cholesterol, this time HDL-derived, at the plasma membrane and the endoplasmic reticulum. In fact, Aster-B is enriched in steroidogenic tissue and its expression is required for storage of HDL-derived cholesterol and steroidogenisis in the adrenal cortex (Sandhu et al., 2018).

Accessible Cholesterol

The involvement of membrane phospholipids in cholesterol transport from the plasma membrane to the endoplasmic reticulum is intriguing and emphasises that a true lipidomic approach is required to unravel the secrets of cell biology. In early studies where the concept of accessible cholesterol was proposed a pool of SM sequestered cholesterol was defined. The difference in accessible and sequestered pools being in the thermodynamic chemical activity, or chemical potential, of cholesterol as a consequence of its membrane environment. When cholesterol is in the regulatory and accessible pool it is available to bind bacterial pore-forming toxins, e.g., perfringolysin O, PFO; anthrolysin ALO (Das et al., 2013, 2014; Infante and Radhakrishnan, 2017), and cholesterol from the SM sequestered pool only becomes accessible to pore-forming toxins after treatment with sphingomyelinase (SMase) at which point it becomes accessible for movement to the endoplasmic reticulum (Das et al., 2014). SMase hydrolyses SM to phosphocholine and ceramide (**Figure 5**). The residual pool of cholesterol does not bind pore forming toxins even after SMase treatment but is essential to maintain membrane morphology (Das et al., 2014).

As mentioned above the GRAM domain of the endoplasmic reticulum resident Aster proteins binds to PS in the plasma membrane in response to cholesterol accumulation, the Aster domain with its StARD-like fold then extracts cholesterol and moves it to the endoplasmic reticulum (**Figure 4**; Sandhu et al., 2018; Ferrari et al., 2020). The reason why GRAM domains recognise PS only in the presence of excess accessible cholesterol is unknown, however, one hypothesis is that an increase in the accessible pool of cholesterol alters the presentation of PS in the inner leaflet of the plasma membrane and facilitates GRAM domain binding and subsequent ASTER domain cholesterol transfer to the endoplasmic reticulum (Ferrari et al., 2020). Importantly, cells deficient in phosphatidylserine synthase 1 (PTDSS1), an enzyme that catalyses the conversion of glycerophosphocholine (PC) to PS (**Figure 5**, central panel), and which are lacking in PS fail to transport LDL-derived cholesterol to the endoplasmic reticulum, which instead accumulates as accessible cholesterol in the plasma membrane (Trinh et al., 2020). This data confirmed the hypothesis that LDL-derived cholesterol leaving the lysosome first moves to the plasma membrane where it

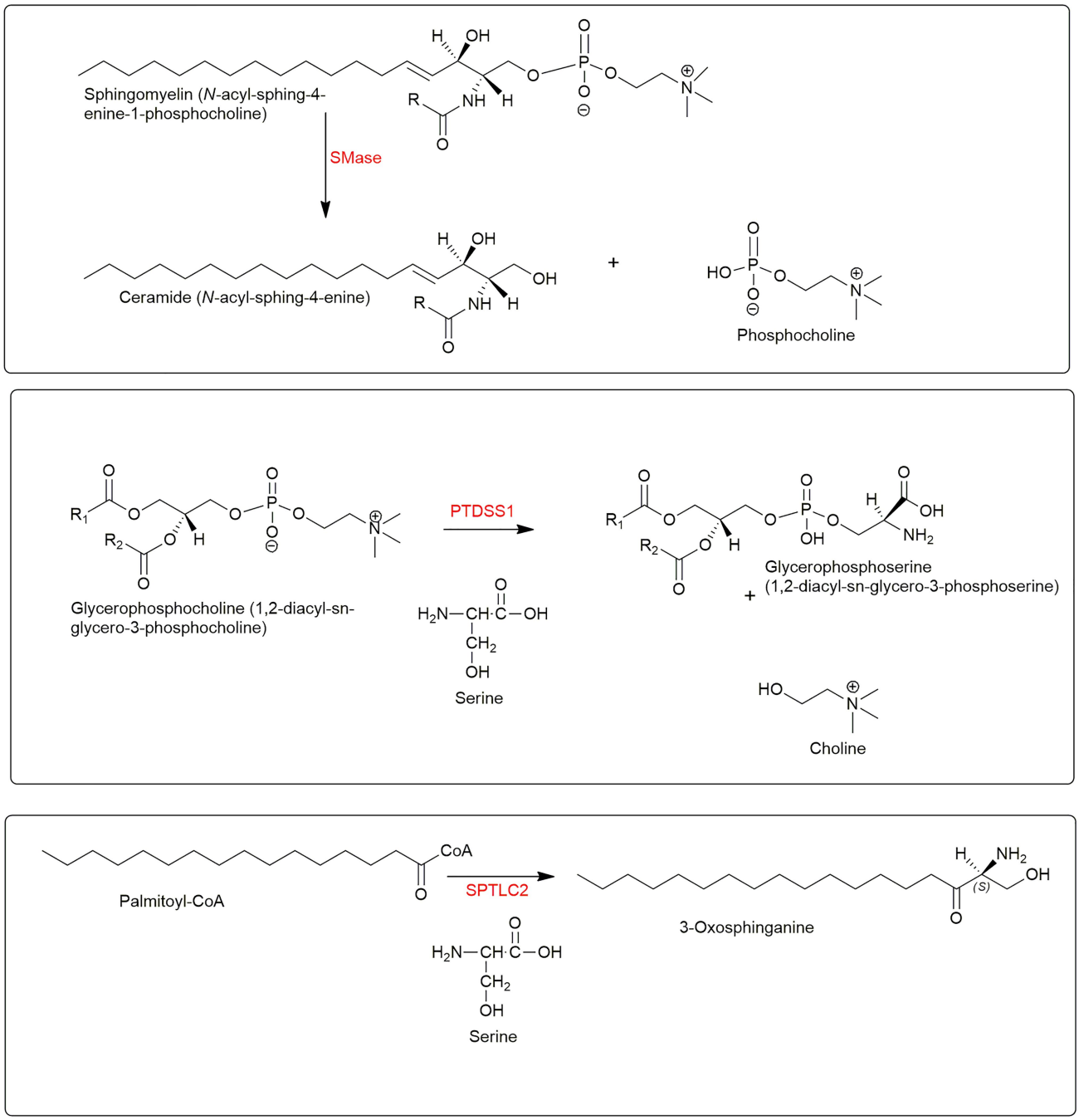

FIGURE 5 | Lipidomoics of accessible cholesterol. **Upper panel**, SMase hydrolyses SM to ceramide and phosphocholine. **Central panel**, PTDSS1 catalyses the conversion of PC to PS. **Lower panel**, SPTLC2 catalyses the formation of 3-oxosphinganine from palmitoyl-CoA and serine.

expands the pool of accessible cholesterol before moving to the endoplasmic reticulum and inhibiting SREBP-2 processing (Trinh et al., 2020).

Remarkably SM and PS have opposite effects on cholesterol transport from the plasma membrane. SM is concentrated in the outer leaflet and sequesters cholesterol while PS is concentrated in the inner leaflet and is essential for cholesterol movement to the endoplasmic reticulum.

Cholesterol Precursors and Metabolites

The SREBP-2 pathway is not only regulated by cholesterol but also by its precursors and metabolites (Radhakrishnan et al., 2007; Spann et al., 2012; Chen et al., 2019). Like cholesterol, desmosterol binds to SCAP and prevents the activation of SREBP-2 to its nuclear form (Radhakrishnan et al., 2007; Spann et al., 2012). Oxysterols when the added oxygen function is on the side-chain (side-chain oxysterols) also inhibit processing

of SREBP-2 to its active form but by binding to INSIG rather than SCAP but still tethering the SCAP-SREBP-2 complex in the endoplasmic reticulum thereby preventing transport of SREBP-2 to the Golgi for activation to its nuclear form (**Figure 2**, lower panel; Radhakrishnan et al., 2007). An alternative route by which the cholesterol precursor lanosterol and side-chain oxysterols regulate cholesterol biosynthesis is *via* degradation of HMG-CoA reductase (Song et al., 2005; Goldstein et al., 2006; Chen et al., 2019). Lanosterol and other 4,4-dimethyl sterols (**Figure 6**) trigger the binding of HMG-CoA reductase to INSIG, this leads to the membrane-embedded E3 ubiquitin ligase gp78 along with the recruited E2 ubiquitin-conjugating enzyme ubc7 to ubiquitinate the reductase which then is extracted from membrane by the ATPase VCP and delivered by to the proteosome for degradation (**Figure 7**, upper panel; Goldstein et al., 2006). The final step is stimulated by geranylgeraniol. The side-chain oxysterol 25-hydroxycholesterol (25-HC) also triggers the binding of HMG-CoA reductase to INSIG (Sever et al., 2003) and its subsequent degradation, and it has been suggested that 25-HC mediates this effect by binding to INSIG, while 4,4-dimethyl sterols may bind to the reductase (Radhakrishnan et al., 2007). Interestingly 27-hydroxylanosterol [more correctly named as (*E/Z*)26-hydroxylanosterol or lanosta-8(*E*),24(*E/Z*)-diene-3β,26-diol (Fakheri and Javitt, 2012)] has equivalent activity to lanosterol but at an order of magnitude lower concentration (Song et al., 2005). Lanosterol does not regulate the SREBP-2 pathway, but other 4,4-dimethyl sterols will repress the processing of SREBP-2 and also stimulate HMG-CoA reductase degradation (Chen et al., 2019).

Besides down-regulation the SREBP-2 pathway cells are protected from cholesterol overload by the endoplasmic reticulum enzyme acyl-CoA cholesterol acyl transferase (ACAT, sterol O-acyltransferase 1, SOAT1) which esterifies cholesterol for storage in lipid droplets. Interestingly, the cholesterol-derived oxysterol, 25-HC, stimulates esterification of cholesterol to its esterified form (Chang et al., 1997; Du et al., 2004).

Liver X Receptors

The liver X receptors (LXRα, NR1H3; LXRβ, and NR1H2) are oxysterol receptors (**Figure 8**; Janowski et al., 1996, 1999; Forman et al., 1997; Lehmann et al., 1997; Fu et al., 2001; Theofilopoulos et al., 2013; Maqdasy et al., 2016). Other endogenous ligands include the cholesterol precursors zymosterol and desmosterol (Yang et al., 2006), C_{27} cholestenoic acids (Song and Liao, 2000; Ogundare et al., 2010; Theofilopoulos et al., 2014), and dendgrogenin A, the histamine conjugate of 5α,6α-epoxycholesterol (**Figure 9**; Segala et al., 2017). The target genes of LXRs code for the cholesterol export proteins ABCA1 and ABCG1, the cholesterol carrier ApoE, the inducible degrader of the LDL-receptor (IDOL), SREBP-1c, and in mouse CYP7A1, the enzyme which catalyses the rate-determining step of the neutral pathway of bile acid biosynthesis (Laffitte et al., 2001; Joseph et al., 2002; Zelcer and Tontonoz, 2006; Zelcer et al., 2009; Wang and Tontonoz, 2018). In addition, the *Gramd1b* gene coding the Aster-B protein is a direct transcriptional target of the LXRs (Sandhu et al., 2018). In combination, activation of LXRs has the overall effect of reducing cellular cholesterol levels. There is considerable cross-talk between SREBP and LXR regulated pathways starting at the level of oxysterols where many side-chain oxysterols activate both LXR and inhibit SREBP processing *via* binding to INSIG (**Figures 2, 9**; Janowski et al., 1999; Radhakrishnan et al., 2007).

INTERFERONS, CHOLESTEROL 25-HYDROXYLASE AND ACCESSIBLE CHOLESTEROL IN PROTECTION AGAINST MICROBIAL PATHOGENS

Interferons and 25-HC

IFNs (IFNs) are cytokines involved in the communication between cells which trigger defence mechanism of the immune system against pathogens including viruses (Blanc et al., 2011, 2013; Liu et al., 2013; Cyster et al., 2014) and bacteria (Bauman et al., 2009; Diczfalusy et al., 2009; Cyster et al., 2014). Type I IFNs, e.g., IFNβ, are produced by many types of cell including macrophages, fibroblasts and endothelial cells and bind to the cell surface IFNα/β receptor (IFNAR) on target cells, while type II IFN (IFNγ in human) produced by T-cells binds to the IFNGR on target cells.

The Toll-like receptor 4 (TLR4) is a pattern recognition receptor (PRR) which is activated by lipopolysaccharide (LPS), a cell wall component of many Gram-negative and some Gram-positive bacteria and also by some viral proteins. Over the last decade links between TLR4, TLR3 (an intracellular PRR), bacterial and viral infections, IFNs, and the oxysterol 25-HC have been uncovered. Activation of macrophage or dendritic cells (antigen-presenting cell) by TLR4 or TLR3 ligands leads to induction of IFNβ, activation of IFNAR and up-regulation of *Ch25h* (*CH25H* in human) the gene encoding cholesterol 25-hydroxylase leading to elevated levels of plasma 25-HC (Bauman et al., 2009; Diczfalusy et al., 2009; McDonald and Russell, 2010; Park and Scott, 2010; Blanc et al., 2013; Liu et al., 2013). *Ch25h*/*CH25H* is now classified as an interferon stimulated gene. 25-HC can be regarded as an immunoregulatory oxysterol; it has a role in the adaptive immune system, suppressing the production of immunoglobulin A (IgA) by B cells (Bauman et al., 2009), it also restrains interleukin-1β (IL-1β) driven inflammation in macrophages by preventing AIM2 (absent in melanoma 2) inflammasome activation (Dang et al., 2017). This effect is achieved by restricting cholesterol biosynthesis by inhibiting the processing of SREBP-2 (Dang et al., 2017). High cholesterol synthesis is required AIM2-dependent inflammasome activation and IL-1β release by macrophages.

Cholesterol-Dependent Cytolysins, Accessible Cholesterol and 25-HC

PFO is a pore-forming toxin secreted by the Gram-positive bacteria *Clostridium perfringens* and is a member of a family of cholesterol-dependent cytolysins (CDC). A mutated version of PFO, PFO*, binds to accessible cholesterol, and at 4°C does not form pores and kill cells (Das et al., 2013). ALO is a closely related CDC, and like PFO*, its cholesterol binding

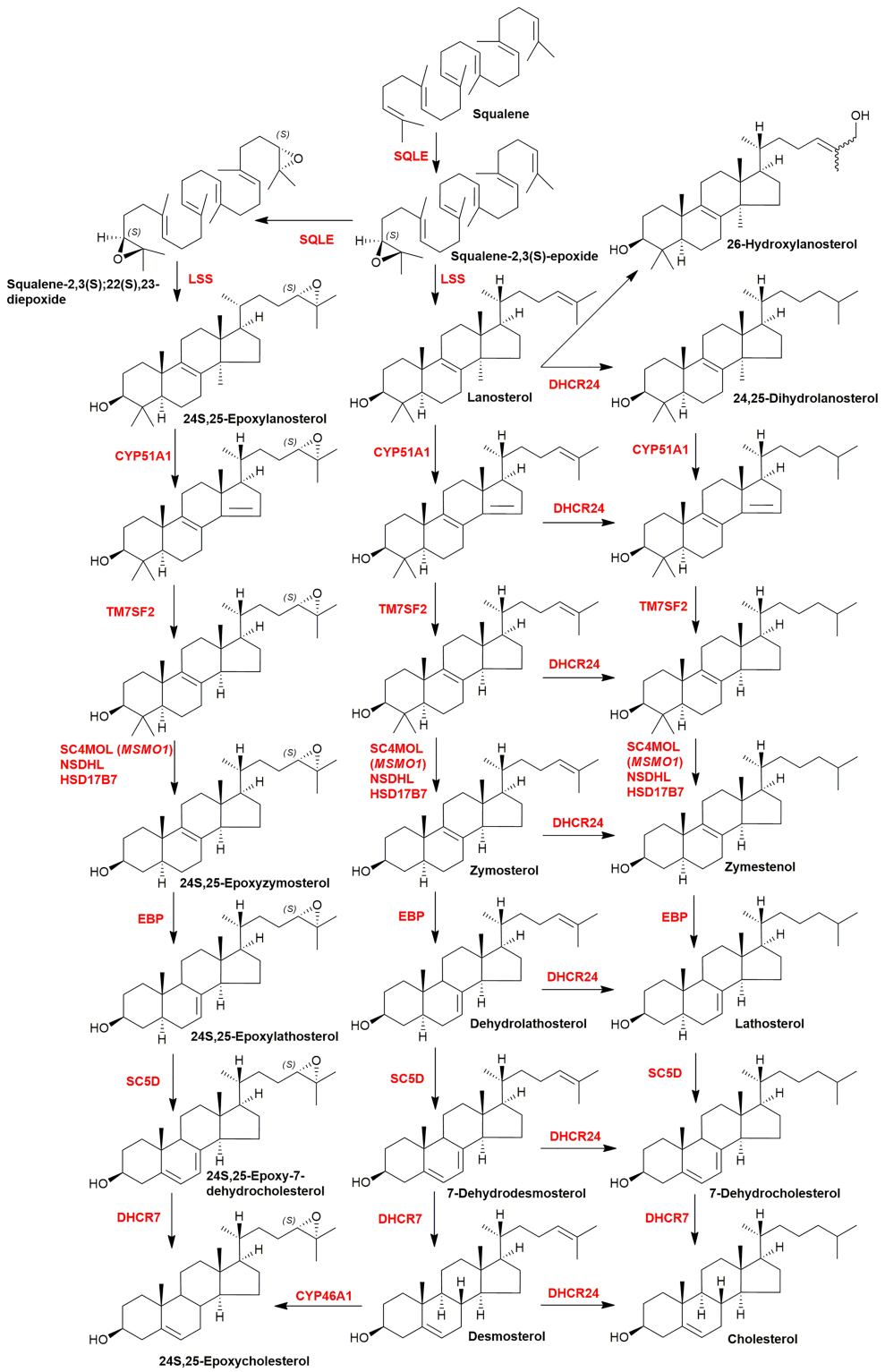

FIGURE 6 | Pathway from squalene to cholesterol and 24S,25-epoxycholesterol. Enzymes are indicated in red.

sub-domain ALOD4, has been used extensively to assess the accessible cholesterol content of membranes (Das et al., 2013, 2014; Infante and Radhakrishnan, 2017; Naito et al., 2019; Ferrari et al., 2020; Trinh et al., 2020). Remarkably, 25-HC protects macrophages and neutrophiles against CDCs including PFO demonstrating an acute interplay between oxysterols and

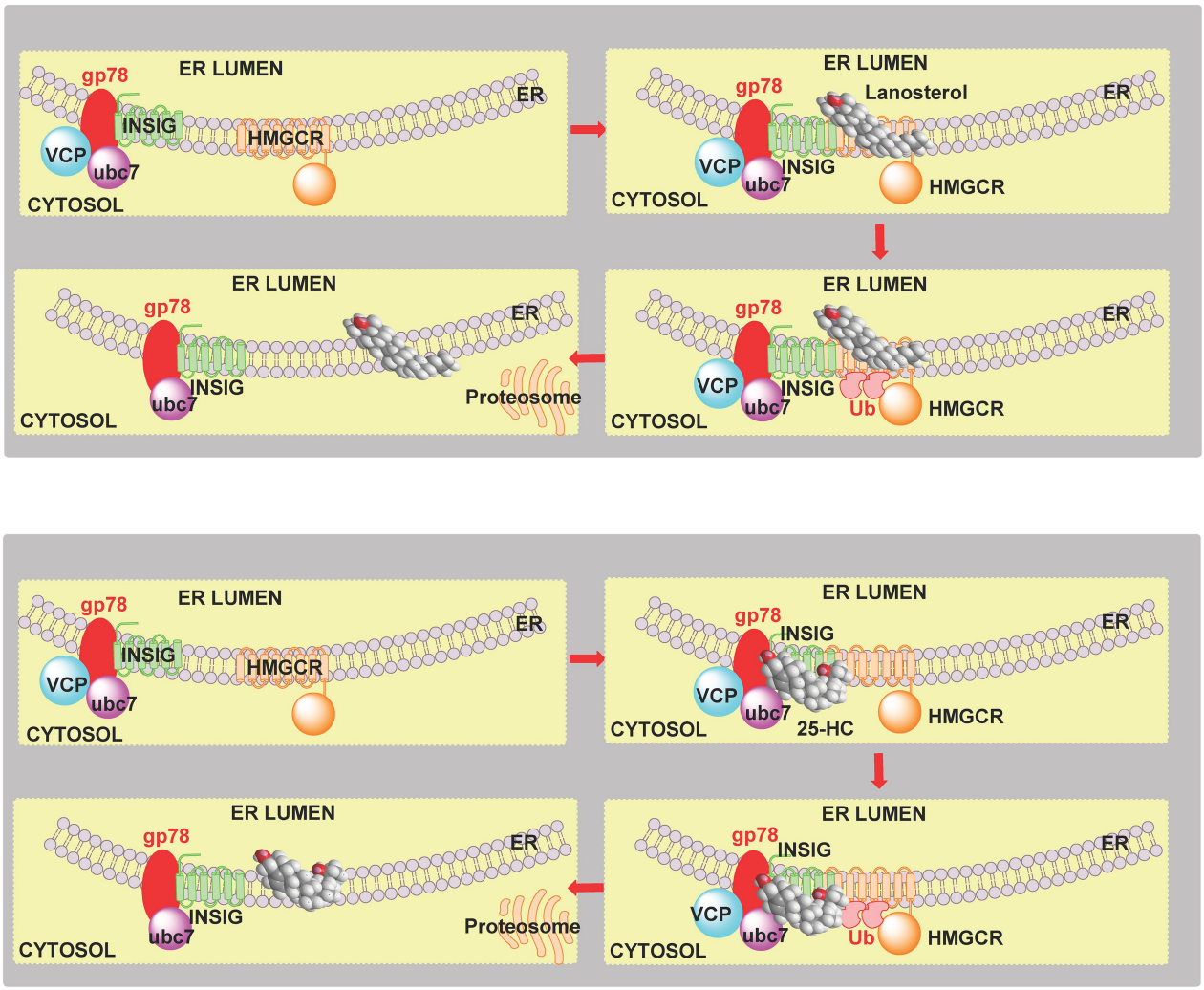

FIGURE 7 | Cartoon representation of the sterol-induced degradation of HMG-CoA reductase. **Upper panel**, 4,4-dimethyl sterols induce the binding of INSIG to HMG-CoA reductase (HMGCR). The E3 ubiquitin ligase gp78 and E2 ubiquitin-conjugating enzyme ubc7 ubiquitinate (Ub) HMG-CoA reductase. In a final step stimulated by geranylgeraniol, ubiquitinated HMG-CoA reductase is extracted from the membrane by VCP and delivered to the proteosome. **Lower panel**, 25-HC similarly induces degradation of HMG-CoA reductase, however, it has been suggested that sterol binding is to INSIG rather than the reductase (Goldstein et al., 2006).

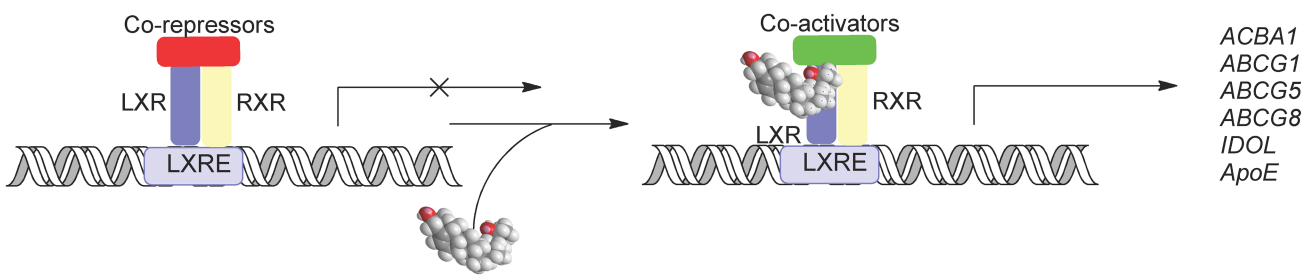

FIGURE 8 | Activation of LXRs by oxysterol. In the absence of ligand LXR-RXR heterodimers bind to the LXR response element (LXREs) and recruit co-repressors and supress gene expression. When activated by oxysterols co-repressors are replaced by co-activators leading to the expression of LXR target genes.

accessible cholesterol (Zhou et al., 2020). The mechanism behind this protection proceeds through activation of PRR on macrophages, IFN-induced expression of the enzyme CH25H and production of 25-HC (Zhou et al., 2020). As discussed above, 25-HC can (i) reduce cholesterol synthesis *via* binding to INSIG and prevent processing of SREBP-2 to

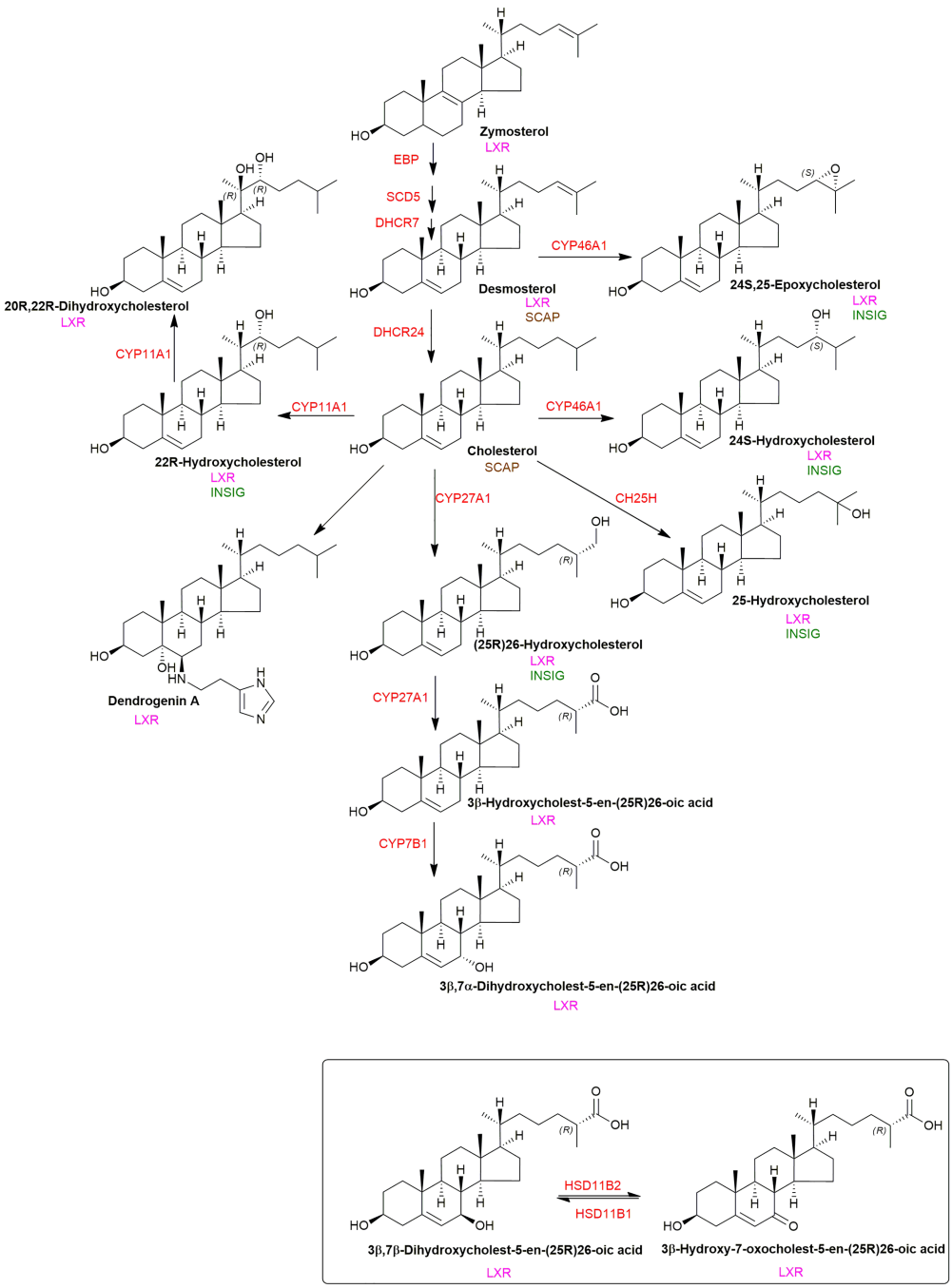

FIGURE 9 | Crosstalk between ligands to LXR, INSIG and SCAP. Enzymes are shown in red. LXR, INSIG and SCAP binding is illustrated where appropriate.

its active form; (ii) induce the degradation of HMG-CoA reductase (Radhakrishnan et al., 2007); (iii) activate the LXRs and induce cholesterol export (Wang and Tontonoz, 2018) and mediate cholesterol ester formation by activation of ACAT/SOAT (Du et al., 2004). It is possible that all four effects contribute to 25-HC mediated protection against CDCs by reducing the pool of accessible cholesterol in the macrophage/neutrophile plasma membrane, and thus lead to reduced CDC binding and pore formation, ultimately reducing CDC toxicity (**Figure 10**; Zhou et al., 2020). Current data suggest that reduction in cholesterol biosynthesis is most important in reducing plasma membrane accessible cholesterol and that, surprisingly, IFN will increase cholesterol ester formation even in the absence of *Ch25h* (Zhou et al., 2020). By maintaining a low pool of accessible cholesterol macrophages and neutrophiles are protected against CDCs, it is likely that 25-HC will also protect endothelium and epithelium cells against pathogen produced toxins *via* a similar mechanism.

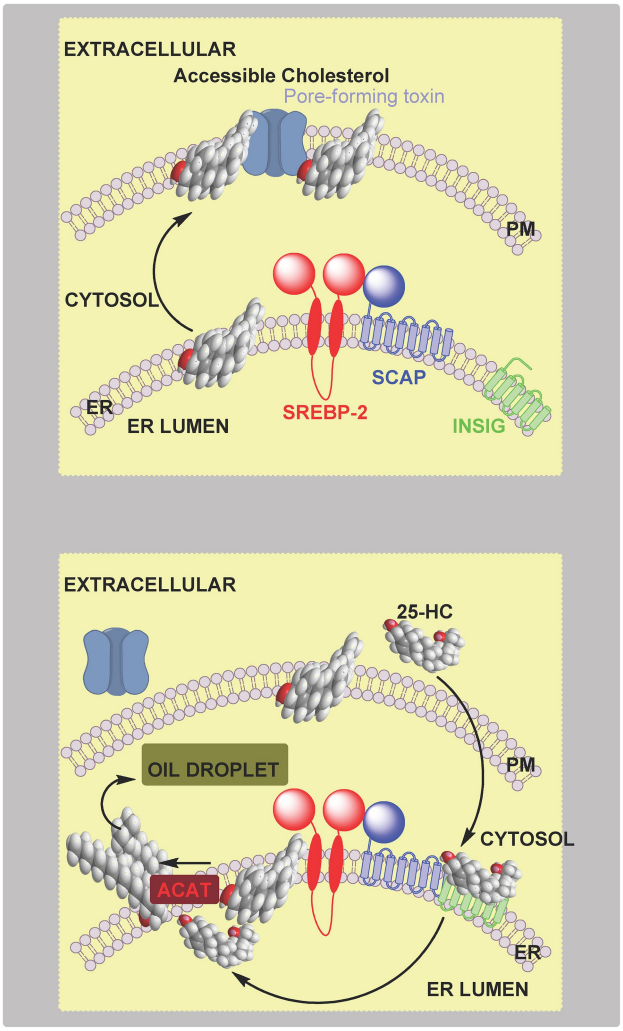

FIGURE 10 | Cartoon representation of the protection by 25-HC of macrophages and neutrophiles against pore-forming toxins. **Upper panel**, pore-forming toxin binds to accessible cholesterol in the plasma membrane, oligomerises and create a pore, ultimately leading to cell death. **Lower panel**, 25-HC generated by macrophages in response to infection rapidly crosses the cell membrane, and (i) inhibits SREBP-2 processing leading to reduced cholesterol synthesis and (ii) activates ACAT/SOAT and cholesterol esterification, in combination leading to a reduction in plasma membrane accessible cholesterol. The consequence is reduced binding of pore-forming toxins to the plasma membrane and protection of the cell. 25-HC can also activate LXR to enhance cholesterol export and can encourage the ubiquitination and degradation of HMG-CoA reductase to repress cholesterol synthesis (not shown).

Bacterial Infection, Accessible Cholesterol and 25-HC

The mucosal epithelium provides a physical barrier between microbial communities and underlying tissue of the host. One way in which the host is protected against microbes is through IFNγ-activated macrophages which communicate with epithelium cells to clear local infections. *CH25H* is one of the hundreds of IFN-stimulated genes which are bactericidal, and it has been shown recently that the enzymatic product of the expressed gene, 25-HC, can prevent in a paracrine fashion, cell–cell transmission in the infected host (Abrams et al., 2020). 25-HC can suppress the contact-dependent cell to cell spread of *Listeria monocytogenes*, a model enteric pathogen, in epithelial tissue. Using ALOD4 to distinguish between pools of plasma membrane cholesterol, 25-HC was found to deplete accessible cholesterol and inhibit bacterial spread and infection, leading to the conclusion that accessible cholesterol is required for bacteria to penetrate adjacent cells (Abrams et al., 2020). Like *L. monocytogenes*, *Shigella flexneri* undergoes cell-to-cell spread *via* membrane protrusions, and like *L. monocytogenes* its spread is inhibited by 25-HC reducing host-cell plasma membrane accessible cholesterol (Abrams et al., 2020). The mechanism by which 25-HC reduces accessible cholesterol in the host epithelium cell was suggested to be *via* activation of endoplasmic reticulum-located ACAT/SOAT, triggering rapid internalisation of accessible cholesterol from the plasma membrane (Abrams et al., 2020). This conclusion was based on data showing inhibition of ACAT/SOAT prevents the removal of accessible cholesterol by the LXR and INSIG ligands 20S-hydroxycholesterol (20S-HC), 25-HC and (25R)26-hydroxycholesterol (26-HC, more commonly referred to as 27-HC; Abrams et al., 2020), however, this data does not rule out LXR activation and inhibition of SREBP-2 processing playing a supporting role in the defence against bacterial spread over a longer time period (**Figure 11**).

Antiviral Activity of 25-HC and Other Oxysterols

25-HC has been shown to have broad antiviral activity against enveloped (Blanc et al., 2013; Liu et al., 2013; Chen et al., 2014) and nonenveloped viruses (Doms et al., 2018; Shawli et al., 2019). In early studies it was shown that in response to viral infection macrophage generated IFN activates the expression of *Ch25h* with the resultant generation and secretion of 25-HC providing both a paracrine and autocrine antiviral response (Blanc et al., 2011, 2013; Liu et al., 2013). This can be achieved by down-regulation of the host cholesterol biosynthesis pathway, consistent with 25-HC repressing the processing of SREBP-2 (Blanc et al., 2013). It is noteworthy, that in one of the early studies performed mostly with mouse cytomegalovirus (MCMV) and bone-marrow-derived macrophages (BMDM) the antiviral activity of 25-HC was increased in lipid depleted conditions where the SREBP-2 pathway is active (Blanc et al., 2013). 25-HC will reduce both the *de novo* synthesis of cholesterol and receptor mediated up-take *via* the LDL-receptor. However, in a different cell model, it was found that over expression of *Srebp-2* in HEK293T cells did not reverse the antiviral effect of 25-HC and instead it was suggested that the antiviral activities of 25-HC were through changing cell membrane properties to inhibit membrane fusion, a requirement to release viral genetic material for subsequent replication (Liu et al., 2013). Other oxysterols are also implicated in the antiviral response (Lembo et al., 2016). *In vitro* studies indicate 26-HC and 24S,25-epoxycholesterol (24S,25-EC) also have antiviral properties, but are less potent than 25-HC (Blanc et al., 2013; Cagno et al., 2017).

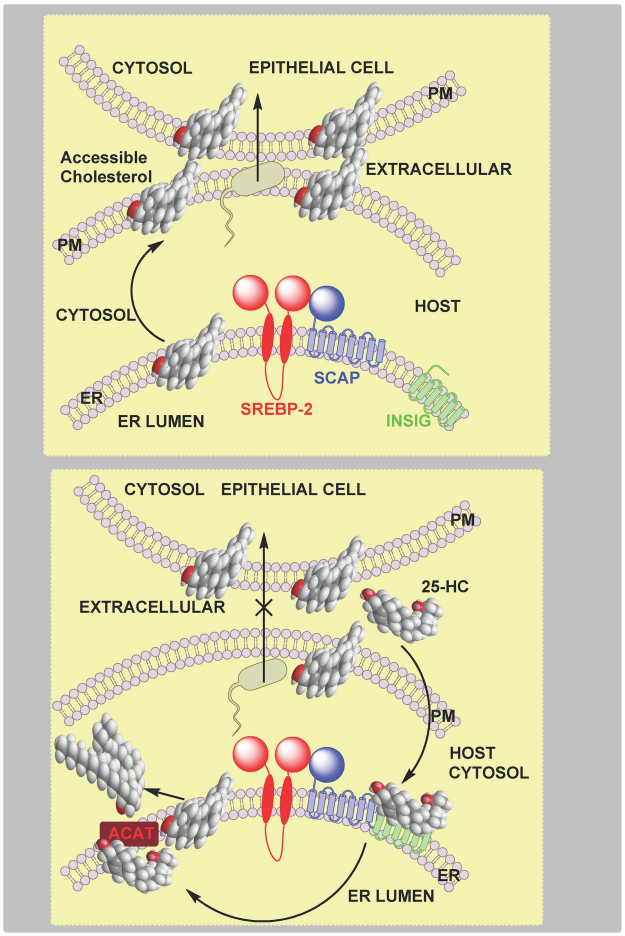

FIGURE 11 | Cartoon representation of the inhibition by 25-HC of bacterial spread between epithelial cells. **Upper panel**, plasma membrane accessible cholesterol is required for bacterial cell–cell transmission. **Lower panel**, activated macrophages secrete 25-HC which stimulates the endoplasmic reticulum-located enzyme ACAT/SOAT to rapidly esterify cholesterol for storage in lipid droplets leading to reduced accessible cholesterol in the plasma membrane, this prevents bacterial spread across neighboring cells. A supporting role in reducing accessible cholesterol is played by inhibition of the SREPB-2 pathway but over a longer timeframe.

25-HC, Severe Acute Respiratory Syndrome Coronavirus-2 and Accessible Cholesterol

IFNs are induced by coronavirus infection (Park and Iwasaki, 2020), and IFN-stimulated genes are up-regulated in severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 infected cells (Lamers et al., 2020). One of these genes, *CH25H*, is found to be up-regulated in macrophages and lung epithelial cells found in bronchioalveolar lavage (BAL) fluid from COVID-19 patients (Wang et al., 2020). Besides infecting lung epithelia cells SARS-CoV-2 also infects intestinal epithelium cells (Lamers et al., 2020; Zang et al., 2020) and *CH25H* was found to be one of the IFN-stimulated genes in primary human enteroids (Zang et al., 2020). In a gene screen performed in HEK293-hACE2 cells (i.e., HEK-293 cells expressing the human ACE2 receptor, the receptor of SARS-CoV and SARS-CoV-2) *CH25H* supressed both SARS-CoV and SARS-CoV-2 pseudo-virus replication (see below; Zang et al., 2020). The product of CH25H enzymatic activity, 25-HC, has been shown to be elevated in some patients suffering from SARS-CoV-2 infection (Marcello et al., 2020; Zu et al., 2020) and 25-HC has been shown to be antiviral against SARS-CoV-2 by blocking spike protein catalysed membrane fusion (Wang et al., 2020; Zang et al., 2020). Besides 25-HC, it should be noted that other oxysterols including 26-HC and 7-oxocholesterol (also called 7-ketocholesterol) have been linked to the antiviral response against SARS-CoV-2 (Marcello et al., 2020; Ghzaiel et al., 2021).

SARS-CoV-2 is an enveloped single stranded RNA virus. It binds to the ACE2 (angiotensin converting enzyme 2) receptor and subsequently infects cells by either a plasma membrane or endosome fusion pathway. The plasma membrane fusion pathway requires the presence of membrane bound TMPRSS2 (transmembrane protease serine 2) to cleave the spike protein for early fusion (Hoffmann et al., 2020). SARS-CoV can enter cells *via* the endosomal pathway using the endosomal cysteine proteases cathepsin to cleave the spike protein, however, the TMPRSS2 mediated pathway may be dominant for SARS-CoV-2 membrane fusion (Hoffmann et al., 2020).

The experimental use of pathogenic SARS-CoV-2 virus demands strict biosafety levels, hence, the use of replication-restricted pseudo-viruses bearing viral coat proteins represent a safe alternative. Vesicular stomatitis virus (VSV), like SARS-CoV-2, is an enveloped virus but only causes mild flu-like symptoms. The VSV envelope G-protein can be replaced by a reporter gene bearing SARS-CoV-2 spike protein, giving, e.g., a VSV-eGFP-SARS-CoV-2 pseudo-virus, appropriate for studying viral entry mechanisms. VSV-SARS-CoV-2 pseudo-viruses have been extensively exploited in two recent studies on the mechanism of 25-HC antiviral activity against SARS-CoV-2 (Wang et al., 2020; Zang et al., 2020).

Focusing on the TMPRSS2 mediated early fusion pathway and human lung epithelial cells, 25-HC was found to inhibit SARS-CoV-2 pseudo-virus at a half-maximal inhibitory concentration (IC_{50}) of 550 nM (220 ng/ml; Wang et al., 2020). Binding of the pseudo-virus to the host cell was not affected, but in a cell model of membrane fusion, 25-HC was shown to block plasma membrane fusion. Based on this data and studies demonstrating the involvement of 25-HC in the reduction of plasma membrane accessible cholesterol (Abrams et al., 2020), it was postulated that 25-HC blocks coronavirus spike-protein mediated membrane fusion by mobilising accessible cholesterol away from the plasma membrane (Wang et al., 2020). This mechanism may also be responsible for the antiviral activity of 25-HC against SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), in addition to SARS-CoV-2 in lung epithelial cells where the plasma membrane fusion pathway is dominant. In experiments using Calu-3 cells, a lung epithelial cell line, where viral entry is *via* TMPRSS2, and using fluorescence-labelled ALOD4 as an indicator of accessible cholesterol, 25-HC was found to deplete accessible cholesterol in a dose dependent manner. Supplementation with cholesterol in complex with cyclodextrin, restored ALOD4 binding (Wang et al., 2020).

Importantly, supplementing cholesterol also restored SARS-CoV-2 pseudo-virus entry in 25-HC treated cells, supporting the idea that 25-HC mobilises plasma membrane cholesterol to inhibit SARS-CoV-2 virus – plasma membrane fusion (Wang et al., 2020). Addition of cholesterol in complex with cyclodextrin also reverses inhibitory effects of 25-HC on Calu-3 cells challenged with SARS-CoV or MERS-CoV pseudo-virus (Wang et al., 2020). The explanation for the reduction of accessible cholesterol in response to 25-HC, may be a explained by (i) activation of LXRs and cellular cholesterol export; (ii) inhibition of SREBP-2 processing leading to a reduction in cholesterol biosynthesis and uptake *via* the LDL-receptor; (iii) stimulation of ubiquitination and proteolysis of HMG-CoA-reductase; and (iv) activation of ACAT/SOAT resulting in enhanced cholesterol ester formation (**Figure 12**). In Calu-3 cells, the depletion of plasma membrane accessible cholesterol resulting from 25-HC treatment is likely to be through activation of ACAT/SOAT, at least in lipid depleted medium, as the ACAT inhibitor Sandoz 58–035, reversed the reduction in accessible cholesterol and rescued SARS-CoV-2 pseudo-virus entry. ACAT/SOAT knockdown by shRNA also enhanced pseudo-virus entry in 25-HC treated cells (Wang et al., 2020). A caveat to these results is that the experiments were performed in lipid depleted medium, so any reduction in LDL-cholesterol uptake as a consequence of 25-HC inactivation of the SREBP-2 pathway leading to down-regulation of the LDL-receptor expression was not considered.

25-HC, SARS-CoV-2, and Endocytosis

An alternative route for coronavirus entry into cells is *via* endocytosis which also requires membrane fusion for viral RNA release (Hoffmann et al., 2020; Tang et al., 2020). 25-HC will inhibit viral replication by blocking the required membrane fusion event (Zang et al., 2020). *In vitro* cell fusion assays confirmed *CH25H* expression blocked membrane fusion, a result phenocopied by 25-HC. Interestingly, fluorescently-labelled 25-HC (C4 TopFluor-25HC), which was found to have an almost identical antiviral activity towards the SARS-CoV-2 pseudo-virus as 25-HC, was found to localise to late endosomes-lysosomes in HEK 293 cells. In addition, 25-HC treatment of these cells led to an accumulation of unesterified cholesterol (Zang et al., 2020). As discussed earlier, NPC1 protein transports unesterified cholesterol to the membrane of the lysosomal compartment, and will also bind 25-HC (Kwon et al., 2009). This data along with evidence that treatment with itraconazole (ICZ) and U18666A, two inhibitors of NPC1, which lead to reduced SARS-CoV-2 pseudo-virus infection, prompted the suggestion that cholesterol accumulation in the late-endosome-lysosome compartment may explain the antiviral activity of 25-HC (**Figure 13**). Importantly, the antiviral activity of 25-HC and ICZ are diminished in serum free medium, indicating that their antiviral activity is dependent on the accumulation of cholesterol in the endosomal-lysosomal compartment (Zang et al., 2020). A caveat to this interpretation of the data is that free 25-HC does not traverse the lysosome, at least not when it inhibits SREBP processing (Kwon et al., 2009). However, fluorescently-labelled 25-HC (C4 TopFluor-25HC) is a version of 25-HC esterified at C-25 through a linker to a fluorescent group, and may behave like other sterol esters and be taken-up by receptor

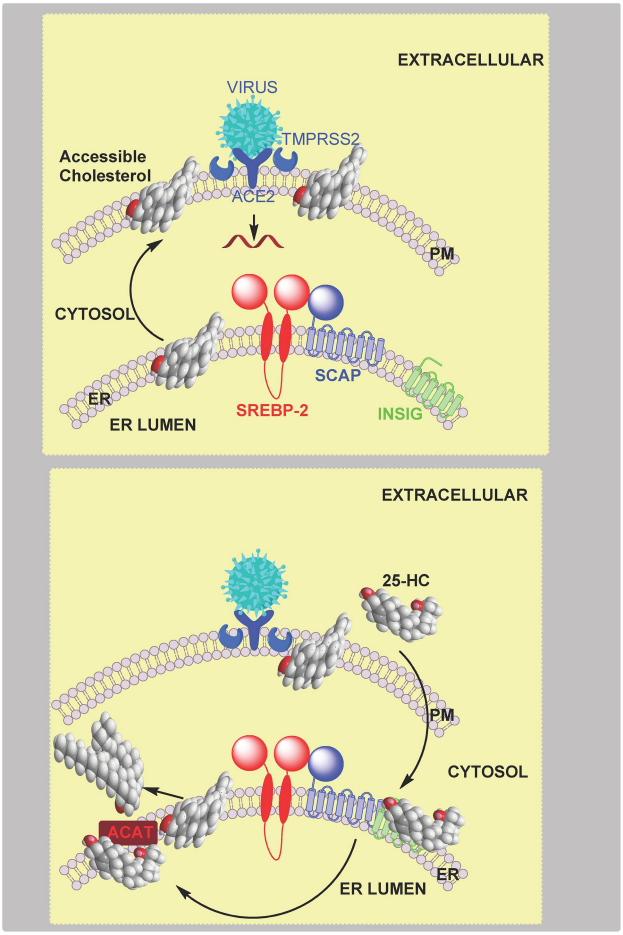

FIGURE 12 | Model of coronavirus entry *via* the early pathway. **Upper panel**, virus binds to the cell surface ACE2 receptor. The membrane-bound TMPRSS2 enzyme triggers the early fusion pathway by proteolytic cleavage of the spike protein to induce the fusion-competent state of the protein. Membrane fusion proceeds and results in release of viral RNA into the cytoplasm. Membrane fusion is dependent on sufficient accessible cholesterol in the plasma membrane. **Lower panel**, 25-HC inhibits membrane-fusion and replication. This is achieved by reducing accessible cholesterol in the plasma membrane. Data from Wang et al. (2020) indicate that this is achieved by activating the endoplasmic reticulum enzyme ACAT/SOAT which esterifies cholesterol. An alternative explanation is 25-HC binding to INSIG and reducing cholesterol biosynthesis and up-take.

mediated endocytosis at the LDL-receptor as part of LDL and enter the lysosome *via* this pathway while free 25-HC could proceed directly to the lysosome if not targeted to the endoplasmic reticulum. In plasma the majority of 25-HC is esterified (Dzeletovic et al., 1995), so if the circulation is the source of antiviral 25-HC then uptake by the LDL-receptor may provide a direct route to the lysosome. Furthermore, both ICZ and U18666A are non-specific inhibitors of NPC1, ICZ also inhibits CYP51, an enzyme in the cholesterol biosynthesis pathway and is an antagonist to Smoothened (SMO) a component of the hedgehog (Hh) signalling pathway (see section Oxysterols, Accessible Cholesterol, and Hedgehog Signalling; Kim et al., 2010). U18666A will also inhibit Aster proteins, important in the transport of accessible cholesterol from

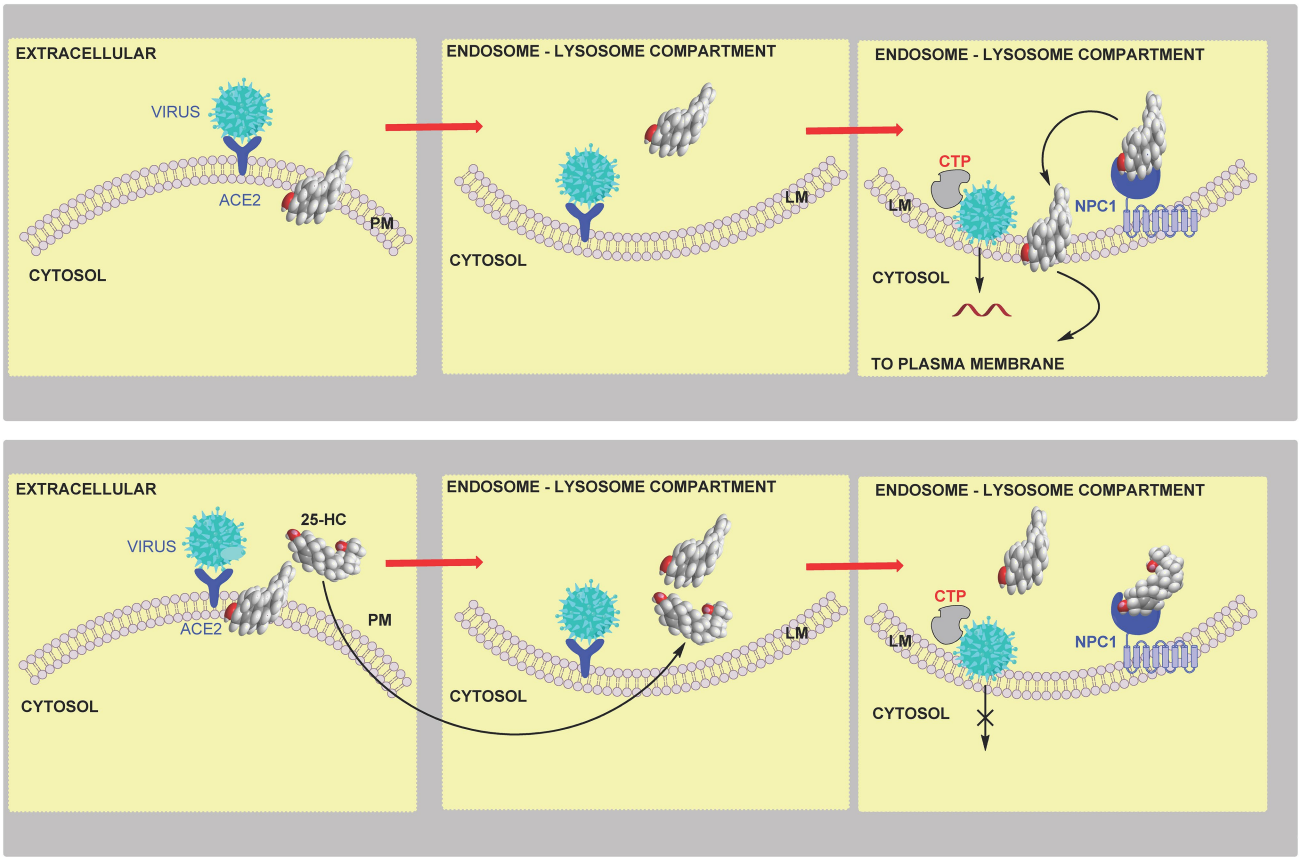

FIGURE 13 | Model of coronavirus entry *via* the late endocytosis pathway. **Upper panel**, in the absence of TMPRSS2, virus is endocytosed and within the endosome-lysosome compartment low pH activates cathepsin (CTP) mediated cleavage of the spike protein triggering membrane fusion and release of viral RNA. **Lower panel**, 25-HC inhibits viral replication by binding to NPC1 and restricting cholesterol transport to the lysosome membrane and export, ultimately preventing membrane fusion and release of viral RNA.

the plasma membrane to the endoplasmic reticulum (Xiao et al., 2021). However, experiments with U18666A were performed with epithelia cells not expressing TMPRSS2, hence mobilising plasma membrane accessible cholesterol would not be required to prevent viral entry in these cells (Zang et al., 2020).

When considered in concert, the results from the two studies (Wang et al., 2020; Zang et al., 2020) lead to a model where SARS-CoV-2 viral infection promotes IFN secretion and the expression of the IFN-stimulated gene *CH25H*. This leads to paracrine or autocrine action of 25-HC leading to inhibition of SARS-CoV-2 spike-protein mediated membrane fusion and inhibition of viral replication. Inhibition of membrane fusion can be mediated by reducing accessible cholesterol at both the plasma membrane and endosome-lysosome membrane level.

OXYSTEROLS, ACCESSIBLE CHOLESTEROL, AND HEDGEHOG SIGNALLING

Oxysterols have numerous roles in biological systems, but the most well defined are those involved in cholesterol homeostasis (Goldstein et al., 2006; Radhakrishnan et al., 2007; Brown et al., 2021; Wang et al., 2021). However, oxysterols have roles beyond cholesterol *per se* and can act as ligands to G protein-coupled receptors (GPCR; Hannedouche et al., 2011; Liu et al., 2011). One GPCR that oxysterols and cholesterol activate is Smoothened (SMO), a critical receptor in the Hedgehog (Hh) signalling pathway (Abdel-Khalik et al., 2020; Radhakrishnan et al., 2020). Like in cholesterol regulation and in protection against microbial infection, oxysterols and accessible cholesterol are closely linked in the Hh signalling pathway.

Oxysterols and the Hedgehog Signalling Pathway

The Hh signalling pathway is an important coordinator of cell–cell communication required during development and regeneration (Radhakrishnan et al., 2020). Defects in the pathway can lead to disorders ranging from birth defects to cancers (Cooper et al., 2003; Raleigh et al., 2018). Hh ligands, e.g., Sonic Hedgehog (SHH) in vertebrates, initiate Hh signalling in a paracrine manner by binding to the extracellular side of Patched 1 (PTCH1), a 12-pass transmembrane protein. A second messenger then communicates the signal to the GPCR SMO,

a 7-pass transmembrane protein, which transmits the signal across the membrane leading to the expression of GLI target genes (Kong et al., 2019; Radhakrishnan et al., 2020). In the absence of Hh ligand, PTCH1 inhibits SMO and prevents Hh signalling (**Figure 14**). The components for Hh signalling are found in primary cilia, antenna-like organelles which are continuous with the plasma membrane (Rohatgi et al., 2007). Side-chain oxysterols are potential second messengers in the Hh pathway, in that they activate Hh signalling in cultured cells, even in the absence of SHH, and induce the accumulation of SMO in primary cilia (Corcoran and Scott, 2006; Dwyer et al., 2007; Nachtergaele et al., 2012; Abdel-Khalik et al., 2020). Side-chain oxysterols bind to the extracellular cysteine-rich domain (CRD) of SMO (Myers et al., 2013; Nachtergaele et al., 2013; Nedelcu et al., 2013; Abdel-Khalik et al., 2020) and have also been found to be enriched in primary cilia (Raleigh et al., 2018). Cholesterol also binds to the CRD of SMO and has been considered as the second messenger between PTCH1 and SMO (Byrne et al., 2016; Luchetti et al., 2016). However, at first thought this is unlikely as cholesterol is so abundant in the plasma membrane. How could changes in cholesterol concentrations necessary for second messenger activity occur without disruption of normal membrane function, and could such changes avoid SREBP-2 regulation of cholesterol levels?

To investigate the nature of the second messengers involved in Hh signalling, a loss of function CRISPR screen was carried out in NIH/3T3 cultured cells targeting lipid-related genes (Kinnebrew et al., 2019). Cells were grown in lipoprotein depleted media and treated with U18666A, to block cholesterol up-take but enhance cholesterol biosynthesis (Kinnebrew et al., 2019). Enzymes of the cholesterol biosynthesis pathway were found to be positive regulators of Hh signalling, but surprisingly enzymes synthesising oxysterols from cholesterol were not found to be positive regulators of Hh signalling. This result needs to be considered in the context that the cultured cells were deprived of cholesterol, so machinery synthesizing oxysterols in these cells is not likely to be activated. An exception is 24S,25-epoxycholesterol (24S,25-EC) which is synthesised in parallel to cholesterol *via* a second epoxidation of squalene by squalene epoxidase (SQLE; **Figure 6**; Nelson et al., 1981). 24S,25-EC is a known activator of Hh signalling and binds to SMO (Raleigh et al., 2018; Qi et al., 2019). However, DHCR24 was found to be a positive regulator of Hh signalling (Kinnebrew et al., 2019), and as this is the one enzyme used to synthesize cholesterol but not 24S,25-EC (**Figure 6**; Nelson et al., 1981), this would suggest that cholesterol is a more likely second messenger between PTCH1 and SMO than 24S,25-EC, but not necessarily if the signalling is paracrine.

While enzymes of the cholesterol biosynthesis pathway were found to be positive regulators of Hh signalling, enzymes of sphingomyelin biosynthesis were found to be negative regulators (Kinnebrew et al., 2019).

Accessible Cholesterol and the Hh Signalling Pathway

In the CRISPR screen, one of the top negative regulators was *Sptlc2* (serine palmitoyltransferase, long chain base subunit 2), which codes for the enzyme catalysing the first committed step in SM synthesis (**Figure 5**, lower panel). The fungal antibiotic myriocin will inhibit SPTLC2 and can be used to deplete SM in cells. Treatment of NIH/3T3 cells with myriocin was found to potentiate Hh signalling, confirming the involvement of SM in the Hh signalling pathway (Kinnebrew et al., 2019). Importantly, mutations in the CRD of SMO which abrogate

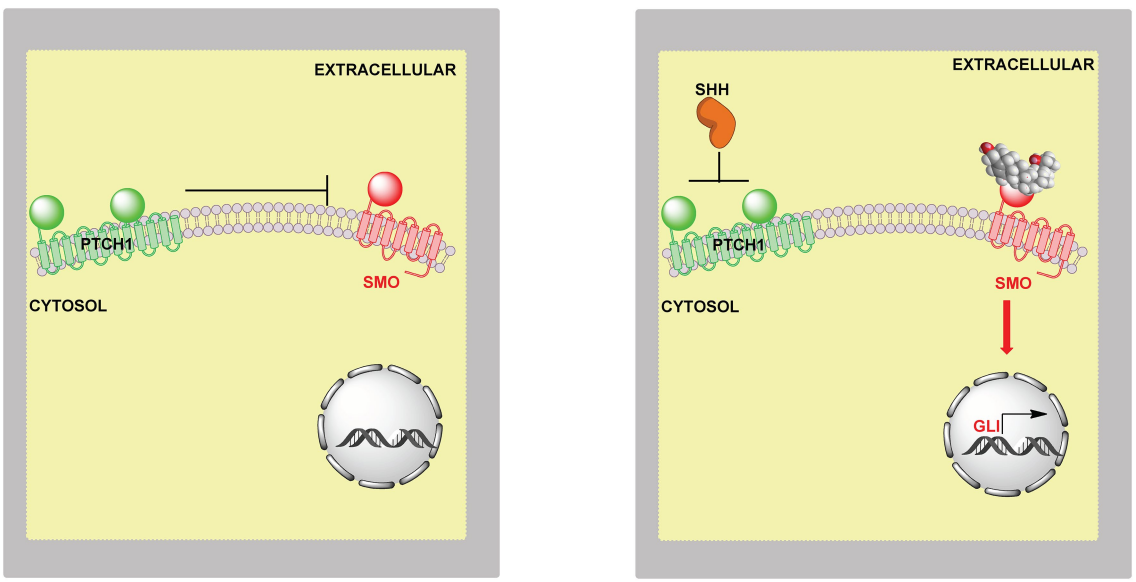

FIGURE 14 | Model of the involvement of oxysterols in Hh signalling. **Left panel**, in the absence of extracellular SHH or oxysterols PTCH1 inhibits SMO and the Hh signal is not transmitted across the membrane. **Right panel**, SHH relives the inhibition by PTCH1 on SMO and oxysterols bind to SMO leading to activation of GLI target genes.

cholesterol binding were found to reduce myriocin driven Hh signalling (Kinnebrew et al., 2019). This supports the concept of cholesterol through binding to SMO acting as the second messenger between PTCH1 and SMO.

The involvement of SM and cholesterol in the Hh signalling pathway suggests the involvement of accessible cholesterol. One model to explain the role SM and cholesterol in Hh signalling is that SM will sequester cholesterol making it inaccessible for Hh signalling thus reducing the availability of accessible cholesterol to act as the second messenger. Evidence to support this model comes from studies with ALOD4, the cholesterol binding domain of the bacterial toxin ALO, that can trap accessible cholesterol found on the outer leaflet of the plasma membrane (Infante and Radhakrishnan, 2017). ALOD4 was found to reduced Hh signalling in cultured cells, in contrast myriocin which blocks SM synthesis was found to enhance Hh signalling (Kinnebrew et al., 2019).

If accessible cholesterol is the second messenger between PTCH1 and SMO how might its regulation be isolated from general plasma membrane cholesterol homeostasis controlled by SREBP-2? One explanation is that the machinery for Hh signalling is located in primary cilia (Rohatgi et al., 2007). Primary cilia have a distinct protein and lipid composition from the bulk plasma membrane to which they merge, including a different SM and accessible content from the rest of the membrane (Nachury and Mick, 2019). Using PFO* as a probe for accessible cholesterol, OlyA [Ostreolysin A, a non-lytic fungal toxin which selectively binds SM sequestered cholesterol (Endapally et al., 2019)] as a probe for SM in complex with cholesterol, and OlyA-E69A (a mutant of OlyA that binds free SM and cholesterol-complexed SM) as a probe for total SM, it was shown that the SM to cholesterol ratio was high in primary ciliary membranes and that myriocin treatment increased the amount of accessible cholesterol relative to the bulk plasma membrane (Kinnebrew et al., 2019). This led to the suggestion that SM in primary cilia is critical for keeping SMO inactive (Kinnebrew et al., 2019). To prevent continuous signalling between PTCH1 and SMO, how might a low level of accessible cholesterol be maintained in primary cilia and how might this be independent from the bulk plasma membrane? One explanation is the "Pump-Leak" model where PTCH1 keeps accessible cholesterol below a threshold for SMO activation by transporting accessible cholesterol out of cilia and on to intra- or extracellular acceptors (Kinnebrew et al., 2019; Radhakrishnan et al., 2020). PTCH1 is a sterol transporter and negatively regulates sterol access to the CRD of SMO (Xiao et al., 2017). When PTCH1 is inactivated by the SHH ligand, accessible cholesterol leaks into the cilia and activates SMO (**Figure 15**, left panel). PTCH1 shows sequence similarity to the cholesterol transporter NPC1 which transports cholesterol out of the lysosome. Both have a sterol sensing domain. NPC1 is suggested to transport cholesterol through a protein tunnel leading to the outer leaflet of the lysosomal membrane (Qian et al., 2020), the soluble protein NPC2 having delivered cholesterol to the N-terminal domain of NPC1. PTCH1 could transport cholesterol in an opposite manner from the outer leaflet of the plasma membrane to a protein acceptor, or alternatively, receive cholesterol from SMO and transport it to the membrane (Radhakrishnan et al., 2020).

It is still not clear how accessible cholesterol gains access to SMO, from the inner or outer membrane leaflet. Besides the

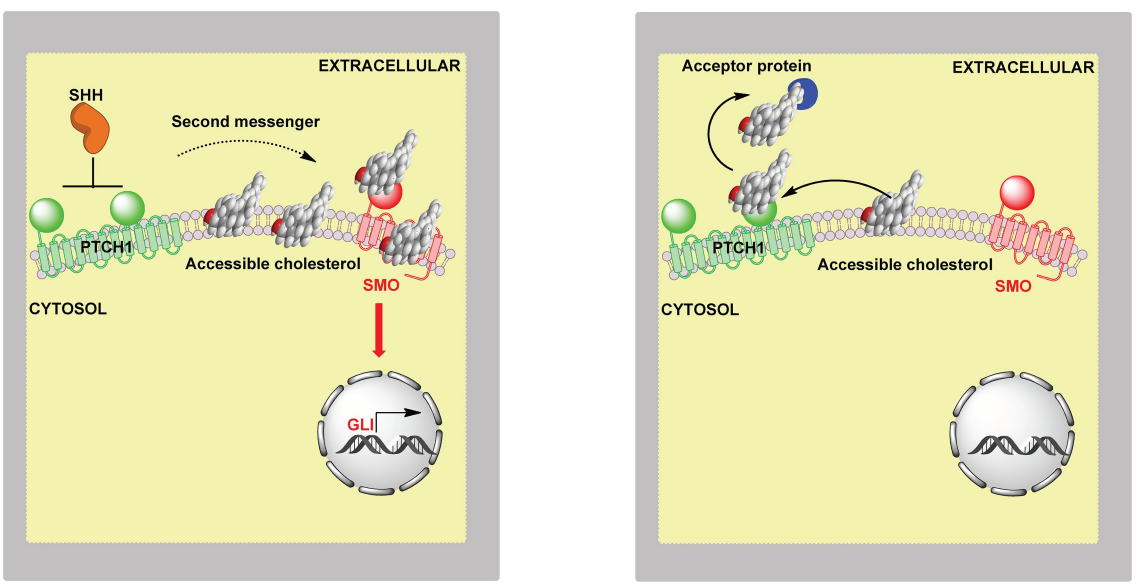

FIGURE 15 | Cartoon representation of the involvement of accessible cholesterol in Hh signalling. **Left panel**, extracellular SHH binds to PTCH1 on the cilium membrane, SMO is activated and GLI transcription factors activate GLI target genes. When SHH binds to PTCH1 it inhibits the action of PTCH1, this may lead to accumulation of accessible cholesterol which acts as the second messenger between PTCH1 and SMO. Cholesterol can bind to the CRD and TMD of SMO, binding at both sites may be required for maximal activation. **Right panel**, the "pump leak" model proposes PTCH1 acts as a sterol pump removing sterols from the membrane in the vicinity of SMO, thereby preventing activation of SMO by sterols. Side-chain oxysterols cross membranes far faster than cholesterol and could potentially occupy one of the sterol binding sites in activated SMO. Oxysterols can activate SMO even in the absence of the SHH ligand.

extracellular CRD (Byrne et al., 2016; Huang et al., 2016), cholesterol has also been shown to bind to the transmembrane domain (TMD) of SMO with access suggested to be from the inner membrane (Deshpande et al., 2019). Probably, PTCH1 inactivation leads to increased accessible cholesterol in both leaflets with cholesterol flip flopping between the two, and either route to SMO activation is possible (Radhakrishnan et al., 2020). In fact, sterol binding to the CRD and TMD may be required for full activation, alternatively one site may be constitutively bound to promote SMO stability while occupation of the second is regulated by PTCH1. A cryo-EM structure of SMO has been solved with 24S,25-EC in the TMD (Qi et al., 2019). Could this be the true regulator of Hh signalling while cholesterol in the CRD is required to promote stability or vice versa?

Back to Oxysterols

Side-chain oxysterols bind to and activate SMO in cultured cells (Corcoran and Scott, 2006; Dwyer et al., 2007; Nachtergaele et al., 2012; Myers et al., 2013; Nedelcu et al., 2013; Raleigh et al., 2018; Abdel-Khalik et al., 2020). Could oxysterols and cholesterol work in concert with one molecule occupying the CRD pocket and the other the TMD and together promote maximum signalling? This is not an unreasonable concept as Smith-Lemli-Opitz syndrome (SLOS) which presents with dysmorphology consistent with defective Hh signalling is accompanied by reduced cholesterol biosynthesis and an unusual pattern of oxysterols. Some of the SLOS derived oxysterols which are modified in both the sterol side-chain and ring fit into the CRD binding pocket of SMO and activate Hh signalling, but are perhaps less efficient activators than simple side-chain oxysterols, resulting in reduced Hh signalling during development of the SLOS embryo compared to that experienced during normal development (Abdel-Khalik et al., 2020). In addition, there is good evidence that oxysterols oxidised in the ring and side-chain activate SMO in the context of medulloblastoma (Raleigh et al., 2018). With regard to the action of oxysterols and cholesterol in Hh signalling it should be noted that activation of LXR by the classic pharmacological ligands TO901317 and GW3965 has been found to inhibit Hh signalling (Kim et al., 2009). TO901317 was found to induce the expression of LXR target genes *Abca1* and *Abcg1* but inhibit the expression of SHH-induced target genes *Ptch1* and *Gli1* (Kim et al., 2009). It is not clear whether LXR activation by oxysterols will have a similar effect on Hh signalling, but if so this may proceed *via* ABCA1 or ABCG1-mediated transport of accessible cholesterol from cilia.

Side-chain oxysterols are often considered as a transport forms of cholesterol, in similar vein they could also be considered as an secondary form of accessible cholesterol in that they are not sequestered by SM (Endapally et al., 2019), they rapidly move through membranes (Lange et al., 1995) and could provide a paracrine form of signalling.

RELATIONSHIP BETWEEN OXYSTEROLS AND ACCESSIBLE CHOLESTEROL

In relation to binding to SMO and activating Hh signalling, side-chain oxysterols can be considered as a rapidly available secondary form of accessible cholesterol. They may be most important with respect to paracrine signalling as they are transferred across membranes orders of magnitude faster than non-esterified cholesterol (Lange et al., 1995; Meaney et al., 2002). This concept of oxysterols as a rapidly available form of signalling accessible cholesterol can be extended further to the regulation of cellular cholesterol by the SREBP-pathway. Side-chain oxysterols will behave like plasma membrane accessible cholesterol and inhibit SREBP-2 processing, the difference being that side-chain oxysterols can move across membranes much quicker than free cholesterol and are thus more likely to have an immediate effect, important for fine tuning of the pathway (Gill et al., 2008).

Importance of Measuring Free and Esterified Oxysterols

During *in vitro* studies oxysterols are added to cells in a non-esterified form, this can be regarded as a mimic of autocrine, paracrine or hormonal signalling. To assess the physiological relevance of the oxysterol, comparisons are made between concentrations required to have a biological effect and those present in a biological tissue or fluid (e.g., plasma). However, confusion can arise if the concentration of oxysterol in the biological material is not clearly defined as being non-esterified or total (esterified plus non-esterified), as it is the non-esterified oxysterol that rapidly crosses membranes. If cells are grown in medium that is replete in lipoproteins, the LDL-receptor will not be expressed, and esterified oxysterols cannot enter cells *via* this route. On the other hand, if cells are grown in lipid depleted medium, then the LDL-receptor will be expressed and oxysterols can be taken up *via* receptor mediated endocytosis along with cholesterol esters. However, release from the lysosome will require prior ester hydrolysis.

CONCLUSION

In this review we have considered the growing importance of the concept of accessible cholesterol in relation to cholesterol homeostasis. The concept can be used to explain how oxysterols are important in defence against microbial pathogens and also how cholesterol itself can act as a second messenger during development. We have paid particular attention to how accessible cholesterol is transported from the plasma membrane to the endoplasmic reticulum. While this system has been well studied, transport of oxysterols in cells has been less extensively studied. In this regard oxysterol binding proteins (OSBP) and the family of OSBP-related (ORP) or OSBP-like (OSBPL), which as their name suggest bind oxysterols, are likely to be important (Olkkonen and Hynynen, 2009).

With respect to oxysterols and the immune system we have discussed how macrophages in response to activation of pattern recognition receptors by pathogens secrete 25-HC, an action also performed by dendritic cells (Bauman et al., 2009; Diczfalusy et al., 2009; McDonald and Russell, 2010; Park and Scott, 2010; Blanc et al., 2013; Liu et al., 2013; Cyster et al., 2014; Dang et al., 2017). 25-HC can have antimicrobial activity

protecting endothelial and epithelial tissue and macrophages and neutrophiles from infection (Abrams et al., 2020; Wang et al., 2020; Zang et al., 2020; Zhou et al., 2020). 25-HC and also 26-HC may be natural antivirals against SARS-CoV-2 (Marcello et al., 2020; Wang et al., 2020; Zang et al., 2020), and we await detailed studies of the oxysterol profiles of infected lung tissue. This will not be easy as lung tissue is highly vascular presenting a problem in distinguishing oxysterols derived from tissue from those present in the circulation. The literature on oxysterol patterns in lung tissue is rather sparse; however, the oxysterol profile of mouse lung has recently been reported following LPS treatment (Bottemanne et al., 2021), while the oxysterol patterns in bronchoalveolar lavage fluid from patients with mild asthma 48 h after acute allergen challenge have been measured (Shen et al., 2017). In human the most well-established oxysterol derivative generated in lung is 3β-hydroxycholestenoic acid (Babiker et al., 1999). Interestingly, 3β-hydroxycholestenoic acid is abundant in the circulation, is an LXR agonist (Song and Liao, 2000), and has been shown to be a potent γ-secretase modulator and could protect against Alzheimer's disease (Jung et al., 2015). The involvement of oxysterols in brain has been discussed elsewhere, but the concept of accessible cholesterol in neuronal development and degeneration has yet to be considered (Wang et al., 2021).

In summary, in this review we have attempted to draw together the concept of signalling *via* accessible cholesterol with that of side-chain oxysterols as paracrine and rapid signalling forms of accessible cholesterol, resurrecting to some extend the oxysterol hypothesis of Kandutsch, Chen and Heiniger (Kandutsch et al., 1978). We have also tried emphasising the protective effects of oxysterols against pathogens and the involvement of oxysterols in modulating accessible cholesterol to provide protection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Plasmalogens and Chronic Inflammatory Diseases

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It is becoming widely acknowledged that lipids play key roles in cellular function, regulating a variety of biological processes. Lately, a subclass of glycerophospholipids, namely plasmalogens, has received increased attention due to their association with several degenerative and metabolic disorders as well as aging. All these pathophysiological conditions involve chronic inflammatory processes, which have been linked with decreased levels of plasmalogens. Currently, there is a lack of full understanding of the molecular mechanisms governing the association of plasmalogens with inflammation. However, it has been shown that in inflammatory processes, plasmalogens could trigger either an anti- or pro-inflammation response. While the anti-inflammatory response seems to be linked to the entire plasmalogen molecule, its pro-inflammatory response seems to be associated with plasmalogen hydrolysis, *i.e.*, the release of arachidonic acid, which, in turn, serves as a precursor to produce pro-inflammatory lipid mediators. Moreover, as plasmalogens comprise a large fraction of the total lipids in humans, changes in their levels have been shown to change membrane properties and, therefore, signaling pathways involved in the inflammatory cascade. Restoring plasmalogen levels by use of plasmalogen replacement therapy has been shown to be a successful anti-inflammatory strategy as well as ameliorating several pathological hallmarks of these diseases. The purpose of this review is to highlight the emerging role of plasmalogens in chronic inflammatory disorders as well as the promising role of plasmalogen replacement therapy in the treatment of these pathologies.

Keywords: plasmalogen, polyunsaturated fatty acids, oxidative stress, inflammation, aging, degenerative disorders, metabolic disorders, plasmalogen replacement therapy

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PLASMALOGENS

Plasmalogens are among the most common glycerophospholipids. These lipids have a broad phylogenetic distribution, being found in many biological membranes (bacteria, protozoa, invertebrates, and mammals) (Braverman and Moser, 2012). In most mammalian membranes they comprise approximately 15 to 20% of total membrane phospholipids (Han et al., 2001;

Abbreviations: Aβ, Beta amyloid peptides; AA, Arachidonic acid; AD, Alzheimer's disease; AGPS, Alkyl-dihydroxyacetone phosphate synthase; AG, Alkylglycerol; AMI, Acute myocardial infarction; BTHS, Barth Syndrome; CAD, Coronary artery disease; CL, Cardiolipin; DHA, Docosahexaenoic acid; DHAP, Dihydroxyacetone phosphate; DHAP-AT, Dihydroxyacetone phosphate acyltransferase; IL-1R, Interleukin-1 receptor; LPS, Lipopolysaccharides; MS, Multiple sclerosis; PC, Phosphatidylcholine; PC-Pls, Plasmenylcholine; PD, Parkinson's Disease; PE, Phosphatidylethanolamine; PE-Pls, Plasmenylethanolamine; PKCδ, Protein kinase C delta; PLA2, Phospholipase A2; PRT, Plasmalogen replacement therapy; PUFA, Polyunsaturated fatty acids; RCDP, Rhizomelic chondrodysplasia punctata; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; TLR, Toll-like receptors; TNFα, Tumor necrosis factor alpha; ZS, Zellweger's syndrome.

Braverman and Moser, 2012). Thus, plasmalogens are among the major lipid components of these membranes. Despite their abundance, plasmalogens had received relatively little attention compared with many other lipid classes over the years. However, lately, plasmalogens have begun to attract more attention due to their association with several degenerative and metabolic disorders as well as aging. In the present review, the focus will be the role of plasmalogens in mammalian inflammatory processes.

Distribution Among Species

Plasmalogens have been found in bacteria, protozoa, invertebrates, and mammals. However, they are not found in plants and, likely, are not present in fungi (Horrocks and Sharma, 1982; Felde and Spiteller, 1994). Among bacteria, they are found in anaerobic bacteria but not in aerobic or facultative aerobic bacteria. The chemical structure of bacterial plasmalogens differs from that of mammals (see below).

Distribution Within the Organism

Plasmalogens are widely distributed within the mammalian organism. They are found in a variety of organs, cells, and other lipid-rich structures such as the myelin sheath and lipoproteins (**Table 1**). The highest amount of plasmalogen is found in the brain, while the liver has the lowest amount of plasmalogens (Koch et al., 2020). Choline plasmalogens (also called plasmenylcholine, PC-Pls) are highly enriched in the heart and smooth muscle, while all other organs are enriched with ethanolamine plasmalogens (also called plasmenylethanolamine, PE-Pls).

Chemical Structure

The Enyl-Ether Linkage

Plasmalogens are a subclass of glycerophospholipids. As such they have a similar chemical structure to diacyl glycerophospholipids. The difference lies in the linkage at the *sn*-1 position of the glycerol moiety (**Figure 1**). While diacyl glycerophospholipids bear an ester bond, plasmalogens present an ether bond. The ether bond links an alkyl chain to the glycerol backbone. Plasmalogens, however, differ from other ether lipids in having a double bond adjacent to the ether linkage, thus making them enyl-ether (vinyl-ether) lipids (**Figure 1**). The enyl-ether linkage imparts differences in the physical, chemical, and biological properties of plasmalogens in comparison to their diacyl counterparts (Nagan and Zoeller, 2001; Braverman and Moser, 2012). From the chemical point of view, the enyl-ether bond is (i) more hydrophobic, (ii) more acid labile, (iii) more oxidation labile, as well as (iv) less involved in hydrogen bonding than their diacyl counterparts (Gorgas et al., 2006).

The Alkyl Chain

The enyl-ether bond links an alkyl chain at the *sn*-1 position of the glycerol moiety in plasmalogens (**Figure 1**). The most abundant alkyl chains in plasmalogens, especially in mammals, are 16 and 18 carbon atoms long (Koivuniemi, 2017; Koch et al., 2020). These chains are, usually, saturated, or monounsaturated. In bacteria, there are also chains with an odd number of carbon atoms, as well as unsaturated alkyl chains at the *sn*-1 position (Nagan and Zoeller, 2001; Řezanka et al., 2011; Farooqui and Horrocks, 2012).

The Acyl Chain

As with their diacyl glycerophospholipid counterparts, plasmalogens bear an acyl chain at the *sn*-2 position of the glycerol backbone attached via an ester bond (**Figure 1**). The predominant acyl chains are 20-22 carbon atoms long and are polyunsaturated (Gorgas et al., 2006; Koivuniemi, 2017). The two most common acyl chains in this position are the polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA, 22:6, an ω-3 fatty acid) and arachidonic acid (AA, 20:4, an ω-6 fatty acid) (Fuchs, 2015; Koch et al., 2020). Plasmalogens from the brain and heart are enriched with DHA, while the colon and spleen are enriched with AA (Koch et al., 2020). In mice, plasmalogens from the kidney are enriched with DHA in males and AA in females (Koch et al., 2020). At the *sn*-2 position, bacterial plasmalogens contains branched and saturated acyl chains (Řezanka et al., 2011).

The Headgroup

The two most common alcohols that are linked to the *sn*-3 position of the glycerol moiety of plasmalogens are choline and ethanolamine. In addition, small amounts of plasmalogens with serine and inositol headgroups have been detected, but only as minor components (Fuchs, 2015). In bacteria, in addition to ethanolamine, serine and glycerol are also found as major components (Řezanka et al., 2011, 2012). Additionally, the plasmalogen form of cardiolipin has also been identified in bacteria (Johnston and Goldfine, 1994).

Physical Properties

Plasmalogens have many effects on the physical properties of biological membranes. For instance, they rigidify membranes, lower the fluidity and stabilize the formation of membrane domains as well as stabilize negatively curved surfaces. All of which have been suggested to contribute to their cellular function.

The enyl-ether linkage has substantial effects on the conformation and dynamics of plasmalogens (Koivuniemi, 2017). For instance, the enyl-ether bond changes the conformation of the lipid headgroup. In PC-Pls this change is reflected in the choline headgroup oriented more toward the water than the bilayer-water interface when compared to its diacyl counterpart (Han and Gross, 1990; Koivuniemi, 2017). The enyl-ether bond also changes the conformation and dynamics of the acyl chain at the *sn*-2 position of the glycerol. It has been shown that this acyl chain in plasmalogens has greater motional freedom compared with its diacyl counterpart. However, in PC-Pls this increased motional freedom is lost at lower temperatures or in the presence of cholesterol (Malthaner et al., 1987).

The enyl-ether bond causes closer packing of the proximal regions of the alkyl-acyl chains in PC-Pls in comparison to its diacyl counterpart (Han and Gross, 1990). Monolayer studies have shown a lower molecular area for PC-Pls compared with the diacyl or alkyl/acyl (plasmanyl, an ether lipid without the enyl-ether double bond) counterparts (Smaby et al., 1983). The differences were less pronounced for PE-Pls. However, the reverse

TABLE 1 | Variation of plasmalogen content among different mammalian organs/structures.

Organ/Cell/ Lipid-rich structure	Total Plasmalogen (% of total phospholipid)	PE-Pls (% of total phospholipid)	PC-Pls (% of total phospholipid)	References
LDL	10	4 (60% of total PE)	4 (4% of total PC)	Bräutigam et al., 1996; Ikuta et al., 2019
HDL	10	5 (55% of total PE)	4 (5% of total PC)	
Brain gray matter	10	10 (49% of total PE)	N.D.	O'brien et al., 1965
Brain white matter	12	12 (86% of total PE)	N.D.	
Myelin	12	12 (90% of total PE)	N.D.	
CNS cell culture	9	8 (49% of total PE)	1 (3% of total PC)	Fitzner et al., 2020
Heart	32	15 (54% of total PE)	17 (42% of total PC)	Hughes and Frais, 1967
Macrophages	15	13 (61% of total PE)	2 (6% of total PC)	Sugiura et al., 1983
Spermatozoa	12	9 (30% of total PE)	3 (9% of total PC)	Poulos and White, 1973

Total = PE-Pls + PC-Pls (other minor plasmalogens not considered).
N.D. = non-detected or trace amounts.
CNS = Central nervous system
CNS cell culture is the average of astrocytes, microglia, neurons, and oligodendrocytes.

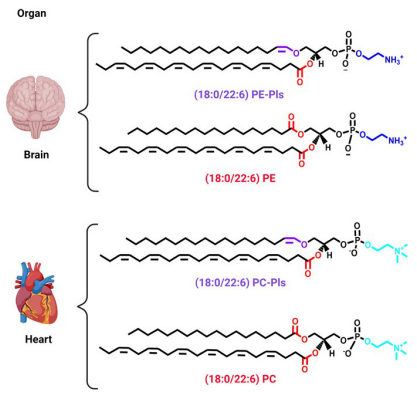

FIGURE 1 | Predominant plasmalogen species in different organs. A cartoon showing two organs, *i.e.*, brain and heart, to exemplify the chemical structure of the predominant plasmalogen species in those organs. Plasmenylethanolamine [18:0/22:6 PE-Pls, 1-(1Z-octadecenyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine] is the predominant plasmalogen species in the brain and plasmenylcholine [18:0/22:6 PC-Pls, 1-(1Z-octadecenyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine] the one in the heart (Koch et al., 2020). The chemical structure of their diacyl counterparts is also shown for comparison. Ethanolamine and choline headgroups are colored in dark and light blue, respectively. The ester and vinyl-ether bonds are colored in red and purple, respectively. The remaining chemical structures are in black. Schematic representations were generated using Biorender (©BioRender - biorender.com).

order was found in molecular dynamic studies (Pietiläinen et al., 2011; Rog and Koivuniemi, 2016). A possible cause for this disagreement is that the monolayer studies were done using plasmalogens with an arachidonoyl group (20:4) at the *sn*-2 position, but the molecular dynamics studies used an oleoyl (18:1) acyl chain. The presence of cholesterol reduced the difference in lateral pressure between the diacyl and enyl-ether lipids. Molecular dynamics showed a high compression at the glycerol backbone for the plasmalogens, resulting in a reduced cross-sectional area of the headgroup (Janmey and Kinnunen, 2006). This results in a slightly thicker bilayer and lower area per lipid molecule.

Simulations also show an increased orientational ordering along the bilayer normal of both the *sn*-1 and *sn*-2 chains in PC-Pls (Koivuniemi, 2017). These chains are more ordered resulting in a more rigid bilayer. This is compatible with plasmalogens being sequestered in liquid-ordered domains in membranes. Indeed, there is evidence that plasmalogens are not distributed uniformly along the plane of the membrane. For instance, they have been shown to be highly enriched in raft-like domains (Pike et al., 2002). Lipidomic analysis has suggested that plasmalogens increase the stability of lipid domains in rat synaptosome membranes (Tulodziecka et al., 2016). Plasmalogens also affect membrane fluidity. Fluorescent probe studies using membranes of *Megasphaera elsdenii* showed that membranes depleted of plasmalogens have a lower order parameter than control membranes (Kaufman et al., 1990). It has been shown that plasmalogen-rich nematode exosomes had increased rigidity compared with murine cells (Simbari et al., 2016).

The enyl-ether linkage is also less compatible with forming hydrogen bonds with water, making the membrane surface more hydrophobic, contributing to the tendency to form inverted phases. This agrees with the studies of Lohner and coworkers showing a lower bilayer to hexagonal phase transition temperature of plasmalogens (Lohner et al., 1984, 1991; Lohner, 1996). Plasmalogens also are enriched in membrane regions with high curvature, such as coated pits, the endoplasmic reticulum (ER), and Golgi cisterna (Thai et al., 2001).

While a considerable understanding of plasmalogen properties in model systems have been gathered, there are certain factors that have not been completely studied. One aspect is the dependence of the physical properties on specific molecular species of plasmalogens. As is described above there are many molecular species of plasmalogens. In many cases

the effects of PC-Pls have been distinguished from those of PE-Pls, but in general, the sensitivity of the physical property to the nature of the acyl chain at the *sn*-2 position has not been studied. In addition, most of the studies have been done *in vitro/in silico* using pure plasmalogens or simple lipid mixtures with only two or at most three lipid components, compared with the complex lipid mixtures found in biological membranes. Furthermore, biological membranes have an asymmetric transbilayer distribution of lipids, which is known to affect membrane properties and their interaction with proteins (Bozelli et al., 2020a). However, most model system studies used symmetrical bilayers that are easier to prepare. Finally, biological membranes have a significant fraction of proteins that can facilitate the formation of membrane domains or can themselves affect membrane physical properties in a manner that would be absent in a model system. Despite these caveats, model system studies have clearly shown differences in the effect of plasmalogens on the physical properties of membranes and how their behavior differs from those of diacyl-lipids or plasmanyl lipids. Changes in membrane physical properties that are specific to plasmalogens must be considered as a possible cause of changes observed in biological membranes resulting from the presence of plasmalogens.

Biological Properties

These manifestations of the effects of plasmalogens on membrane physical properties have been proposed to play a role in a variety of biological functions. Because of the chemical lability of the enyl-ether bond, plasmalogens are suggested to be protective agents against oxidation, acting as scavengers of radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Reiss et al., 1997; Hahnel et al., 1999a; Zoeller et al., 2002). The ability to scavenger ROS/RNS is ascribed to the oxidation-labile enyl-ether bond. *In vitro* plasmalogens decrease the oxidative degradation of PUFA with an efficacy like vitamin E (a mitochondrial antioxidant used to prevent lipid peroxidation). Plasmalogens terminate lipid peroxidation since the products of plasmalogen oxidation are unable to further propagate oxidative reactions (Maulik et al., 1994; Sindelar et al., 1999; Khan et al., 2008; Dott et al., 2014). *In cellula,* plasmalogens increase the resistance of cells to oxidative stress (Zoeller et al., 2002). In addition, in brain white matter from cerebral adrenoleukodystrophy patients, plasmalogen levels are inversely correlated with ROS levels, *i.e.*, increased ROS leads to a decrease in plasmalogen (Khan et al., 2008). Likewise, administering plasmalogen precursors, which increased plasmalogen levels, to a rat model of reperfusion injury, reduced lipid peroxidation (Maulik et al., 1994). Furthermore, increasing plasmalogen levels protects human endothelial cells during hypoxia (Zoeller et al., 2002).

The formation and properties of lipid domains is under active investigation (Bozelli and Epand, 2018). Lipid domains are thicker than the surrounding membrane and they are in the liquid-ordered state that would favor the presence of plasmalogens. It is generally agreed, however, that these domains, in addition to plasmalogens, are also enriched in cholesterol and sphingomyelin and that these domains are of small size and are transient. There is also considerable evidence that lipid domains play an important role in many signal transduction pathways (Bozelli and Epand, 2018). Signal transduction can also occur due to the production of plasmalogen-derived lipid messengers, such as AA. The finding that lipid domains also have a high content of plasmalogens suggests a mechanism for their role in inflammation (Brites et al., 2004). Thus, in addition to the direct effects of plasmalogens on membrane physical properties, it is also likely that plasmalogens have a role in signal transduction (Dorninger et al., 2020).

Negative curvature lipids, particularly PE-Pls, increase the rate of fusion and stimulate extracellular and intracellular vesicle trafficking, particularly for synaptic vesicles (Glaser and Gross, 1994, 1995). There is also evidence that PE-Pls plays a role in the fusion of enveloped viruses with cell membranes (van Meer et al., 2008). This is the case for cytomegalovirus and influenza virus (Liu et al., 2011; Gerl and Sampaio, 2012). The membranes of some parasites are also enriched in plasmalogens (Brouwers et al., 1998; Villas Bôas et al., 1999; Simbari et al., 2016). Exosomes of parasites also contain high levels of plasmalogens in their membranes as do extracellular vesicles from platelets (Pienimaeki-Roemer et al., 2013). Plasmalogens in these vesicles may increase the rate of membrane fusion, however, the sequestration of plasmalogens in lipid domains may alter certain signaling pathways. Feeding PC-3 cells, a metabolic precursor of plasmalogens, hexadecyl-glycerol, causes a large increase in the release of exosomes (Phuyal et al., 2015). Plasmalogens cause the number of caveolae to be reduced and their size becomes smaller, and this lipid also affects axonal sorting and myelin formation (Gorgas et al., 2006; da Silva et al., 2014).

The small change in the chemical functional group from an ester to an enyl-ether, found in plasmalogens, has a dramatic impact on the effect of plasmalogens on membrane physical properties. Hence, both the chemistry, and physical properties of plasmalogens contribute to their role in a variety of cell biology phenomena.

METABOLISM OF PLASMALOGENS

Biosynthesis

The *de novo* biosynthesis pathway of plasmalogens comprises enzymatic reactions that take place on both peroxisomes and ER (**Figure 2**) (Nagan and Zoeller, 2001; Wanders, 2014). The biosynthesis of PE-Pls is the best characterized one. Most evidence suggests that PC-Pls is formed from PE-Pls via headgroup transfer and/or remodeling and, therefore, both lipids have common biosynthetic pathways. PE-Pls biosynthesis is initiated in the peroxisome where dihydroxyacetone phosphate (DHAP) is esterified with acyl-CoA by a matrix peroxisomal enzyme, dihydroxyacetone phosphate acyltransferase (DHAP-AT) (Nagan and Zoeller, 2001; Wanders, 2014). The next enzyme in the pathway for the biosynthesis of PE-Pls, namely alkyl-DHAP synthase (AGPS), catalyzes the replacement of the fatty acid with a fatty alcohol attached to the *sn*-1 position via an ether bond. DHAP-AT and AGPS form a heterotrimeric complex, which is believed to facilitate substrate channeling. Both these

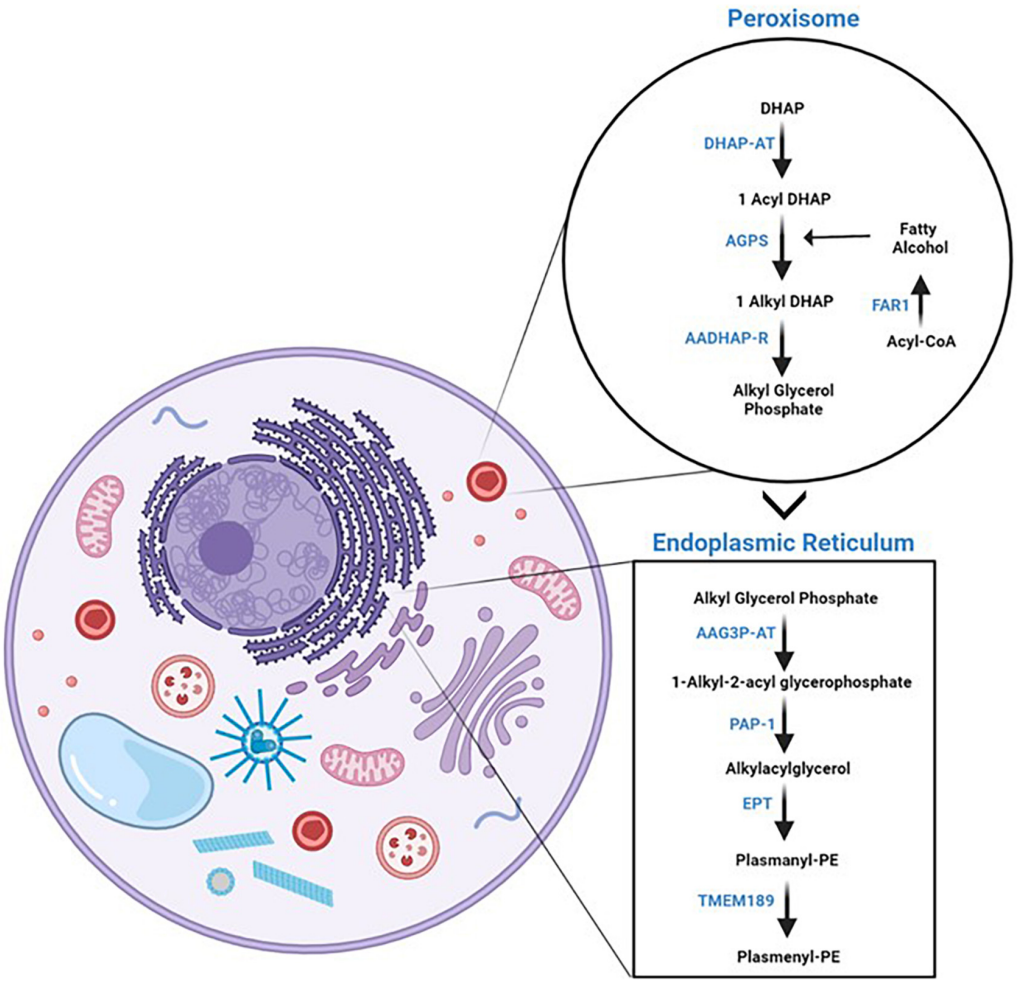

FIGURE 2 | Plasmalogen *de novo* biosynthesis. On the left panel, a cartoon of a cell with a focus on the organelles where plasmalogen *de novo* biosynthesis takes place, *i.e.*, peroxisomes and endoplasmic reticulum. On the right panel, the enzymatic reactions of plasmalogen *de novo* biosynthetic pathway are separated by the organelles where they take place. The reader is referred to the text for the names of the enzymes. Schematic representations were generated using Biorender (©BioRender - biorender.com).

enzymes are imported into the peroxisomal matrix via peptide signals in their sequences, which depend on transmembrane peroxisomal transporter pathways. The fatty alcohol is provided by a fatty acyl-CoA reductase, Far1, which is anchored to the cytoplasmic face of the peroxisomal membrane. The third reaction is catalyzed by an enzyme found at both peroxisome and ER and is a common point between the biosynthesis of both plasmalogens and diacyl phospholipids. This enzyme, acyl/alkyl-DHAP reductase (AADHAP-R) forms 1-alkyl-2-lyso-*sn*-glycero-3-phosphate by reducing the ketone at *sn*-2 position. All the remaining reactions take place in the ER (Nagan and Zoeller, 2001; Wanders, 2014). The first reaction at the ER is catalyzed by the lysophosphatidate acyltransferases (AAG3P-AT), which links an acyl chain from acyl-CoA to the position *sn*-2 of the glycerol moiety. In the following step, phosphatidate phosphohydrolase 1 (PAP-1) removes the phosphate of 1-alkyl-2-acyl-*sn*-glycero-3-phosphate. Next, phosphoethanolamine (coming from CDP-ethanolamine) is attached to the hydroxyl group at position *sn*-3 of the glycerol moiety by the enzymatic action of ethanolamine phosphotransferase, EPT, yielding plasmanyl-PE. From that, PE-Pls is formed via oxidation catalyzed by an ER desaturase to form the vinyl double bond. Recently, the gene that encodes plasmenylethanolamine desaturase in humans has been identified as transmembrane protein 189 (TMEM189) (Werner et al., 2020).

Plasmalogen biosynthesis has been proposed to be regulated by the modulation of the rate-limiting reaction, that is, the one catalyzed by Far1. This is supported by the findings that Far1 levels (but not those of DHAP-AT, AGPS, or AADHAP-R) are elevated in plasmalogen-deficient cells (Honsho et al., 2010; Honsho and Fujiki, 2017; Kimura et al., 2018). That is, plasmalogen levels are regulated by a negative feedback mechanism. It has been shown that Far1 protein levels decrease at normal plasmalogen levels but increase significantly upon decrease in plasmalogen content (Honsho et al., 2010; Honsho and Fujiki, 2017; Kimura et al., 2018). This is not a result

of a change in Far1 expression, but rather in the rate of degradation of Far1, which is increased via a mechanism dependent on posttranslational modification (Honsho et al., 2017). It has been recently proposed that the levels of Far1 are regulated by sensing the content of PE-Pls in the inner leaflet of the plasma membrane in cultured cells (Honsho et al., 2017). Bypassing the peroxisomal reactions by administration of a plasmalogen precursor, an alkylglycerol (AG), increased plasmalogen biosynthesis and induced the degradation of Far1 (Nagan et al., 1997; Nagan and Zoeller, 2001; Honsho et al., 2008, 2010, 2013; Bozelli et al., 2020b). Administration of AG to young rats shows restoration of plasmalogen levels in all tissues but the brain (Das et al., 1992). The regulation of the biosynthesis in tissues is less well characterized. While it is reasonable to propose that the regulation of plasmalogen synthesis in tissues would occur by a negative feedback mechanism, as the one reported in cultured cells, in tissues there is the possibility that the regulation might involve other mechanisms.

Degradation

The steady state levels of plasmalogens are a result of their rate of biosynthesis and degradation. In the brain, one of the organs with highest PE-Pls content, it seems that there are two pools of PE-Pls. In white matter, PE-Pls are mainly found in the myelin sheath where its content is kept at a relatively constant level (Rosenberger et al., 2002). In gray matter, PE-Pls present a high turnover rate with a half-life of *ca.* 20 min. There are several ways that could lead to the degradation of plasmalogens, these include (i) removal of headgroup, (ii) oxidation of the enyl-ether bond, and hydrolysis of the (iii) alkyl chain and (iv) acyl chain.

Degradation of plasmalogen could occur by removal of the headgroup by a phospholipase C or D. It has been shown that PE-Pls can be the substrate of a phospholipase C, which yields 1-alkenyl-2-acyl-*sn*-glycerol (Wolfs et al., 1985). While the action of phospholipase D on PE-Pls yields 1-alkenyl-2-acyl-*sn*-phosphatidic acid (plasmenylphosphatidate) (van Iderstine et al., 1997). The enyl-ether bond can also be a site of action for plasmalogen degradation. It has been reported that the enyl-ether bond is sensitive to radical attack (by ROS and RNS) upon oxidative stress (Nagan and Zoeller, 2001; Zoeller et al., 2002). The major products of radical attack are eicosatetraenoic acid hydroxylated, 2-monoacylglycerol phospholipid, pentadecanol, formic acid, α-hydroxyaldehyde of various chain lengths, 1-formyl-2-arachidonoyl glycerophospholipid, and lysophospholipid (Gorgas et al., 2006). In addition, the enyl-ether bond can be attacked by cytochrome c upon oxidative stress leading to the formation of α-hydroxy fatty aldehydes and 2-arachidonoyl-lysophospholipid (Jenkins et al., 2018). Finally, plasmalogen can be degraded by the action of phospholipases A2 (PLA2), which cleaves the acyl chain at the *sn*-2 position yielding free fatty acid and lysoplasmalogen (Yang et al., 1996). Since plasmalogens are usually enriched with PUFA at the sn-2 position, which themselves are lipid bioactive molecules, the action of PLA2 has received special attention. There has been the identification of plasmalogen-selective PLA2, including one from rat pancreas, which is selective for AA (Hazen et al., 1990; Ford et al., 1991; Yang et al., 1996, 1997). Lysoplasmalogens may, then, be further degraded by lysoplasmalogenases or reacylated to restore plasmalogens (Arthur et al., 1986; Jurkowitz-Alexander et al., 1989; Jurkowitz-Alexander and Horrocks, 1991).

CHRONIC INFLAMMATION AND PLASMALOGEN

Inflammation is an important immune response that our body uses to protect itself from infection and injury (Medzhitov, 2008). Inflammation localized inside the brain and spinal cord is generally described as neuroinflammation (**Figure 3**) (DiSabato et al., 2016). Dysregulation of inflammation leads to chronic inflammation. Chronic inflammation has been identified as a common element in pathophysiological conditions where plasmalogens levels were reported to be decreased.

The inflammatory pathway is coordinated by complex regulatory networks that rely on signals coming from 4 distinct functional groups, those are: the (i) inducer (initiate the cascade of both cellular and molecular events), (ii) sensor (activated by the inducer), (iii) mediator (produced upon sensor activation), and (iv) effector (tissues/organs with functional states altered by the mediator to elicit the desired inflammatory response). For

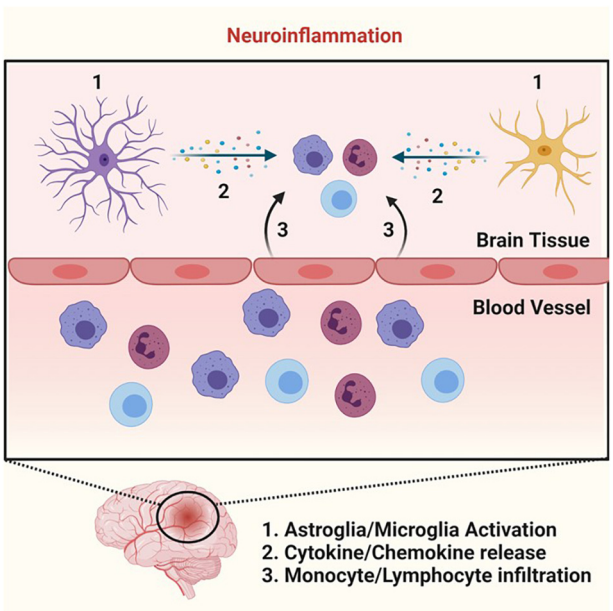

FIGURE 3 | Neuroinflammation. Cartoon summarizing the key events that lead to neuroinflammation. Neuroinflammation begins with microglia and astroglia activation (step 1) that stimulates the release of various cytokines and chemokines (step 2). The cytokine and chemokine production leads to the recruitment of monocytes and lymphocytes and their subsequent infiltration into the parenchyma (step 3), allowing these immune cells to perform their necessary functions in the process of neuroinflammation. Schematic representations were generated using Biorender (©BioRender - biorender.com).

instance, in the case of neuroinflammation triggered by bacterial infection, receptors of the innate immune system (such as Toll-like receptors, TLRs – the sensor) on microglia and astrocytes recognize bacteria (the inducer) (**Figure 3**) (Medzhitov, 2008). Toll-like receptors activation leads to a coordinated cascade of events via the production of mediators, which culminates in the recruitment of leukocytes (such as monocytes and lymphocytes – the effectors) at the site of infection (**Figure 3**) (Zhou et al., 2006). In this process, various mediators are produced including cytokines, chemokines, and lipid mediators that will allow leukocytes to become activated to fight the pathogen at the infection site. Once the pathogens are defeated by the immune system, there is a switch from pro-inflammatory to anti-inflammatory response (the resolution phase). This simplified sequence of events depicts a successful acute inflammatory response (Medzhitov, 2008). However, if the acute inflammatory response fails, a chronic inflammatory state develops.

Lipid mediators are crucial signaling molecules involved in the inflammatory response and its resolution (the return of tissue to homeostasis). Hence, any dysregulation in the production of these lipid mediators could lead to chronic inflammation and excessive tissue damage, which, in turn, would lead to a disease state (Fullerton et al., 2014). Several lipid mediators are derived from the metabolism of PUFA such as AA, DHA, and eicosapentaenoic acid (EA, ω-3, 20:5). For instance, oxidation of AA to produce prostaglandins, thromboxanes, and leukotrienes are involved in pro-inflammatory response, while the metabolism of AA to yield lipoxins, and that of DHA and EA to yield resolvins, protectins, and maresins are involved in the anti-inflammatory response (Denisenko et al., 2020). These PUFA are essential fatty acids and need to be obtained through diet. However, they are not found in the body in their free acid form, rather esterified to glycerophospholipids. Plasmalogens are predominantly enriched with AA and DHA at the *sn*-2 position of the glycerol moiety and, therefore, they are proposed to play a role in inflammation by acting as reservoirs of these important lipid mediators. For example, up to 40% of the PE-Pls in macrophages and neutrophils (immune cells important in the inflammation process) contain AA at the *sn-2* position, which constitutes 75% of AA in these cells (Sugiura et al., 1983; Kayganich and Murphy, 1992). Upon lipopolysaccharide (LPS) stimulation (an inflammatory inducer), plasmalogens from macrophage cells are prone to hydrolysis to release AA to produce pro-inflammatory eicosanoids (Gil-de-Gómez et al., 2017).

Plasmalogens could also contribute to inflammation via modulation of membrane physical properties. For instance, plasmalogens have been shown to be critical in determining proper membrane fluidity and lipid domain formation for efficient signal transduction events (Rubio et al., 2018). In the brains of Alzheimer's disease mice models, it has been reported that plasmalogens function by modulating TLR4 endocytosis and, consequently, decreasing the production of inflammatory cytokines, which, in turn, reduces the inflammatory phenotype (Ali et al., 2019). It was proposed that this role was due to either inhibition of clathrin-dependent endocytosis and/or enhancement of caveolin/lipid raft-mediated endocytosis (Cai et al., 2013; Ali et al., 2019). In addition, lysoplasmalogens (produced via the hydrolysis of the acyl chain at the *sn*-2 position of plasmalogens) have been proposed to actively participate in the inflammatory process via promotion of neutrophil adherence to the endothelium (White et al., 2007). PE-Pls containing AA can also act as an intermediate in the production of anandamide (arachidonoyl ethanolamine, an endocannabinoid), which has anti-inflammatory properties mediated by binding to cannabinoid receptors in the brain (Rettori et al., 2012). Anandamide can also serve as a reservoir of AA and, therefore, the production of eicosanoids (Cravatt et al., 1996). It, thus, seems reasonable that a decrease in plasmalogen levels could impair the inflammatory response by a variety of molecular mechanisms ranging from specific interactions to secondary effects on membrane physical properties.

LOWERED PLASMALOGEN LEVELS IN PATHOPHYSIOLOGICAL CONDITIONS

Recently, there has been an increased attention devoted to plasmalogens. This is a consequence of the identification that in several pathophysiological conditions the levels of plasmalogens are altered. In conditions ranging from aging to degenerative and metabolic disorders it has been shown that the levels of plasmalogens are decreased. However, it is not completely understood at present the molecular mechanisms governing the decrease of plasmalogen levels in these pathophysiological conditions. In this section we will discuss the plasmalogen-related changes in some of these conditions.

Aging

Aging in humans is accompanied by a host of molecular and cellular changes that could lead to impaired cell function and organ failure, which, in turn, could trigger degenerative processes (Giorgi et al., 2018). For instance, as one ages mitochondrial function decreases and oxidative stress increases (Boss and Seegmiller, 1981; Bektas et al., 2018; Giorgi et al., 2018; Liguori et al., 2018). In addition, in older individuals chronic inflammation develops (Ferrucci and Fabbri, 2018). Several mechanisms lead to inflammatory processes upon aging including genetic, cellular malfunction, and oxidative stress caused by defective mitochondria (Boss and Seegmiller, 1981; Bektas et al., 2018; Ferrucci and Fabbri, 2018; Giorgi et al., 2018; Liguori et al., 2018). Plasmalogen levels also change as a function of human age (**Table 2**). Plasmalogen content in newborns is extremely small (*ca.* 7% of the total phospholipid mass in the brain) (Farooqui et al., 2008). In the first year of life there is a dramatic increase (8-fold) in PE-Pls in brain white matter (Farooqui et al., 2008). Plasmalogen levels keep increasing linearly up to 30–40 years of age and then, at 70 years of age a significant (linear) decrease in their levels is observed (Rouser and Yamamoto, 1968). Indeed, with elderly individuals (70 years of age), it has been shown that PC-Pls and PE-Pls content in the serum (present within lipoproteins for transport from the liver to other organs) dropped 40% in comparison to healthy young controls (Maeba et al., 2007). This correlation between decreased plasmalogen levels and aging was also confirmed in studies

TABLE 2 | Plasmalogen-related abnormalities in different pathophysiological conditions.

	Control	Aging	RCDP	ZS	AD	PD	MS	BTHS	CAD
Plasmalogen Biosynthesis	N	N	D	D	D	N	N	N	N
Plasmalogen Degradation	N	D	N	N	D	D	D	D	D
Proposed mechanism of plasmalogen content change	-	Increased turnover Increased oxidative stress	RCDP1: Defect in PEX7 gene RCDP2: Lower activity in DHAP-AT RCDP3: Lower activity in AGPS	Absence of peroxisomes	Decreased peroxisomes in neurites Increased oxidative stress	Increased oxidative stress	Increased oxidative stress MS-related demyelination	Increased expression of iPLA2β	Increased oxidative stress
Reported plasmalogen content	-	Approximately 40% lower in elderly	Variable decrease in plasmalogen content depending on disease severity	Near absence plasmalogens	40 mol% loss in white matter 10 mol% loss in gray matter 30 mol% loss in gray matter in severe dementia	20% - 60% plasmalogen loss	General decrease in plasmalogen content	10% - 28% ethanolamine plasmalogen loss. Highest decrease seen in liver (28%) and BTHS lymphoblasts (25%)	General decrease in plasmalogen content
Plasmalogen species affected	-	Both ethanolamine and choline plasmalogens affected	Primarily ethanolamine plasmalogens affected	Both ethanolamine and choline plasmalogens near absent	Primarily ethanolamine plasmalogens affected	Primarily Ethanolamine plasmalogens affected	Both choline and ethanolamine plasmalogens affected	Choline plasmalogens affected in heart Ethanolamine plasmalogens affected in other organs	Primarily choline plasmalogens affected
Reference	-	Terlecky et al., 2006; Maeba et al., 2007; Jenkins et al., 2018	Itzkovitz et al., 2012; Dorninger et al., 2014; Noguchi et al., 2014	Itzkovitz et al., 2012; Dorninger et al., 2014; Noguchi et al., 2014	Ginsberg et al., 1995; Han et al., 2001; Igarashi et al., 2011; Sachdev et al., 2013	Fabelo et al., 2011; van Horssen et al., 2019; Mawatari et al., 2020; Patergnani et al., 2021	Fabelo et al., 2011; van Horssen et al., 2019; Mawatari et al., 2020; Patergnani et al., 2021	Kimura et al., 2018, 2019; Bargiela and Chinnery, 2019	Meikle et al., 2011; Christodoulidis et al., 2014; Sutter et al., 2016

An outline of different pathophysiological conditions where plasmalogens levels have been reported to be decreased and the proposed effect on plasmalogen biosynthesis, degradation, and mechanism of plasmalogen content change. RCDP, Rhizomelic chondrodysplasia punctata; ZS, Zellweger's syndrome; AD, Alzheimer's disease; PD, Parkinson's disease; MS, Multiple sclerosis; BTHS, Barth syndrome; CAD, Coronary artery disease.

examining mammalian tissue (Brosche and Platt, 1998). While there is no present explanation for the molecular mechanisms leading to lower plasmalogen levels upon aging, it has been noted that aging cause lower plasmalogen levels by either impairing the biosynthesis and/or increasing degradation (likely, caused by oxidative stress) (Terlecky et al., 2006; Jenkins et al., 2018).

Peroxisomes Diseases

Peroxisomes are organelles that play an important role in the metabolism of lipids and radical species (ROS/RNS) and, therefore, are modulators of a variety of signaling pathways dependent on them, including inflammation and immune response (Fransen et al., 2017). Peroxisomes *de novo* biogenesis emerges from a hybrid of mitochondrial and ER-derived pre-peroxisomes (Sugiura et al., 2017). Furthermore, peroxisomes act in concert with mitochondria in several metabolic processes (Sugiura et al., 2017). Therefore, it is not unexpected that dysfunction in one organelle tends to affect the other. Peroxisome diseases are the name given to the collection of pathologies caused by mutations in the genes encoding proteins involved in either peroxisomes biogenesis or function. Since, peroxisomes are the place where the *de novo* biosynthesis of plasmalogens is started, it is not surprising that in peroxisome diseases there is a decrease in plasmalogen levels. In mice models of peroxisomal deficiency diseases, neuroinflammation is an established feature, suggesting that peroxisomes play an important role against degeneration and inflammation in the brain (Kassmann et al., 2007). Below a discussion of the plasmalogen-related changes in two peroxisomal deficiency diseases will be made.

Zellweger's Syndrome

Zellweger's Syndrome (ZS) is a rare autosomal recessive disorder characterized by a defective peroxisome biogenesis, a consequence of mutations in one of the 13 PEX genes that are responsible for peroxisome formation and function (Steinberg et al., 2020). In ZS, peroxisomes are deficient, mitochondria dysfunctional, oxidative stress increased, and there is neuroinflammation (Heymans et al., 1983; Baumgart et al., 2001; Kassmann et al., 2007). In post-mortem tissue of infants with ZS, plasmalogen levels are decreased significantly in comparison to controls (**Table 2**) (Heymans et al., 1983, 1984). The extent of decrease varies with tissue and could be as low as 10% of that found in controls. In the brain, kidney, and liver of ZS patients PE-Pls is the plasmalogen species affected, while in muscle and heart PC-Pls is the species affected (Heymans et al., 1983, 1984).

Rhizomelic Chondrodysplasia Punctata

Rhizomelic Chondrodysplasia Punctata (RCDP) is a rare autosomal recessive disorder characterized by defective plasmalogen biosynthesis, a consequence of mutations in peroxisomal enzymes involved in this pathway (**Table 2**) (Bams-Mengerink et al., 2013). The most common type of RCDP (RCDP1) has been associated with defects in the PEX7 gene, that encodes a peroxisome import receptor responsible for the proper targeting of PTS2-proteins into peroxisomes (Itzkovitz et al., 2012). Defects in enzymatic function or expression levels of peroxisomal enzymes responsible for initiating plasmalogen synthesis, DHAP-AT and ADHAP-S, are also associated with less frequent RCDP2 and RCDP3, respectively (Itzkovitz et al., 2012; Noguchi et al., 2014). The severity of RCDP phenotype seems to correlate with plasmalogen content in fibroblasts derived from RCDP patients (Dorninger et al., 2014). For a non-severe RCDP, a 40% reduction in PE-Pls content was reported, while that value increased to more than 70% in the severe phenotype (Dorninger et al., 2014). Hence, in the case of RCDP it has been proposed that the low plasmalogen levels might be responsible for the symptoms of RCDP (Braverman et al., 2002; Itzkovitz et al., 2012; Bams-Mengerink et al., 2013; Duker et al., 2017).

Neurodegenerative Disorders

Neurodegenerative disorders are diseases that involve the deterioration of the brain due to the progressive loss of structure and/or function of neurons, which might lead to cell death. Diseases that occur because of neurodegeneration include Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple sclerosis (MS) (Gitler et al., 2017). Mitochondrial dysfunction and oxidative stress are key players in neurodegeneration (van Horssen et al., 2019; Patergnani et al., 2021). In neurodegeneration, mitochondrial-derived RNS/ROS can trigger inflammation as well as stimulate immune signaling cascades to intensify the inflammatory process (Heneka et al., 2014; Patergnani et al., 2021). In addition, one observation that has started to gain increased interest is the fact that in several neurodegenerative disorders a marked decrease in plasmalogen levels has been reported. This opens a new and exciting avenue of research in the field of neurodegenerative disorders. This section will expand on the relationship between plasmalogen loss and different neurodegenerative disorders.

Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles, amyloid-β plaques, synaptic loss, and abnormal Tau proteins in the brain (Ginsberg et al., 1995; Guan et al., 1999; Han et al., 2001). These molecular changes result in progressive memory loss alongside mitochondria dysfunction and oxidative and inflammatory damage to the brain (Ginsberg et al., 1995; Guan et al., 1999; Han et al., 2001; Braverman and Moser, 2012). Post-mortem analyses of the brains of AD patients have shown a decrease in PE-Pls and PC-Pls in both gray and white matter of their brains (**Table 2**) (Ginsberg et al., 1995; Igarashi et al., 2011). At the earlier stage of the disease, the loss of plasmalogen in AD patients is higher in white matter (40 mol%) than in gray matter (10 mol%) (Han et al., 2001). As the disease progresses to severe dementia, the gray matter plasmalogen loss increases to around 30 mol% (Han et al., 2001). Given that AD is primarily a disease of gray matter, a positive correlation between disease progression and plasmalogen loss is seen (Han et al., 2001; Sachdev et al., 2013). However, the correlation between plasmalogen loss and AD has been questioned by a recent study where it has been shown a lack of correlation between low plasmalogen levels and the ApoE4, a biomarker of AD (Han, 2005; Braverman and Moser, 2012).

Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disease characterized by the presence of fibrillar aggregates of α-synuclein within Lewy bodies and the associated loss of dopaminergic cells within the basal ganglia of patients, which leads to motor function impairment (Olanow et al., 2009; Miller and O'Callaghan, 2015; Powers et al., 2017; Bozelli et al., 2021). The progression of PD is associated with dysfunctional mitochondria, increased oxidative stress, and neuroinflammation (van Horssen et al., 2019; Patergnani et al., 2021). Recent literature examining PD patients has identified the presence of altered plasmalogen levels (**Table 2**). Although not as excessive of a decrease as in AD, ethanolamine head group-containing ether lipids decreased 30% in both plasma and erythrocytes of PD patients (Mawatari et al., 2020). It has been proposed that in PD, plasmalogen loss at lipid domains from cortical gray matter could lead to impaired cellular signaling (Fabelo et al., 2011).

Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system (Huang et al., 2017). Believed to be an autoimmune disorder, it occurs due to infiltration of autoreactive lymphocytes across the blood brain barrier into the central nervous system (Trapp and Nave, 2008). Autoreactive lymphocyte invasion leads to localized inflammation, demyelination, axonal loss, and gliotic scarring (Trapp and Nave, 2008). In MS, mitochondrial dysfunction drives neuroinflammation, likely via an oxidative stress mechanism (Bargiela and Chinnery, 2019). While the plasmalogen-specific literature surrounding MS is inchoate, recently a marked decrease in plasmalogen (PC-Pls and PE-Pls) content in the serum of MS patients experiencing both remission and relapse of MS has been reported (**Table 2**) (Ferreira et al., 2021). It has been proposed that this decline in plasmalogen species in MS patients might have various causes, including (i) increased immune system stress contributing to the reduction of plasmalogen via its oxidation, and (ii) MS-related demyelination, which might also contribute to plasmalogen loss as the myelin sheath is enriched in plasmalogen species (Ferreira et al., 2021).

Heart Diseases

Heart diseases are the first cause of death in western countries. These are a group of conditions that affect the structure and function of the heart, which could arise due to different molecular and cellular events. The heart is an organ that relies heavily on aerobic metabolism and, therefore, mitochondrial dysfunction plays a crucial role in many heart diseases (Martín-Fernández and Gredilla, 2016). Mitochondria dysfunction can increase oxidative stress, which can activate the inflammasome and lead to chronic inflammation in cardiometabolic diseases (Patergnani et al., 2021). Inflammation and oxidative stress have been proposed to play a role in the initiation, progression, and complications of cardiometabolic diseases (Tousoulis et al., 2008). Recently, the involvement of plasmalogen in heart diseases has started to emerge; specifically, the decrease in PC-Pls, which is the main plasmalogen in the heart and could constitute up to 40 mol% of the total choline phospholipids (Heymans et al., 1983; Diagne et al., 1984; Kimura et al., 2016). In this section, a discussion of plasmalogen-related changes in a couple of heart diseases will be made.

Barth Syndrome

Barth Syndrome (BTHS) is a rare genetic disorder, which mainly affects the heart, but also muscles, the immune system, and growth. BTHS is characterized by mutations in tafazzin, a phospholipid-lysophospholipid transacylase that is involved in the last step of the *de novo* biosynthesis of the mitochondrial-specific lipid cardiolipin (CL) (Barth et al., 1996; Bione et al., 1996; Vreken et al., 2000; Schlame et al., 2003). Barth Syndrome patients present altered content and molecular species of CL as well as abnormal mitochondrial structure and function (Vreken et al., 2000; Schlame et al., 2003; Valianpour et al., 2005; Xu et al., 2005; Gonzalvez et al., 2013; Wang et al., 2014; Goncalves et al., 2021). Moreover, a link has been reported between inflammation and mitochondria in the pathology of BTHS (Wilson et al., 2012). While alterations in CL and mitochondria have been the focus in BTHS research, lately it has been acknowledged that there are more widespread lipid changes in BTHS. A particularly important one is the observation that plasmalogen levels decreased markedly in several organs (brain, heart, and liver) of a tafazzin knockdown mouse model of the disease as well as in lymphoblast cells derived from BTHS patients (**Table 2**) (Kimura et al., 2018, 2019). The changes in plasmalogen are much higher than those observed for CL. In the heart, PC-Pls is the plasmalogen affected, while in brain, liver, and lymphoblasts derived from BTHS patients PE-Pls is the lipid species affected most (Kimura et al., 2018, 2019). The exact reason for this PC-Pls deficiency is unknown; however, it has been suggested that it might be related to the observed increase in the expression of $iPLA_2\beta$ (a calcium-independent phospholipase A2 that is plasmalogen-selective), in the hearts of tafazzin knockdown mice (Kimura et al., 2018).

Coronary Artery Disease

Coronary Artery Disease (CAD) is the most common type of heart disease. It is caused by the development of atherosclerotic plaques (lipid deposits, mainly cholesterol) inside arterial walls over time, which could end up in occlusion and, consequently, acute myocardial infarction (AMI) (Sutter et al., 2016). While lipid accumulation has been the major focus of the research on plaque formation and destabilization, more recently literature has emphasized the key roles of chronic inflammation, mitochondria dysfunction, and oxidative stress on these processes (Christodoulidis et al., 2014). Previous data has shown that lipids such as cholesterol, glycerophospholipids, sphingolipids, and triacylglycerols are important risk factors for atherogenesis (Sutter et al., 2016). Plasmalogens have also been reported to be altered in CAD (**Table 2**). It has been reported that PC-Pls levels are decreased in the plasma of CAD patients, specifically four molecular species of PC-Pls (33:1, 33:2, 33:3, and 35:3) were reduced (Meikle et al., 2011; Sutter et al., 2016).

As illustrated by the discussion above, the recent interest in plasmalogens by the scientific community is not surprising. While the relationship between plasmalogen loss and these

various pathophysiological conditions is clear, there is a lack of understanding of the molecular mechanisms. However, in some of these conditions a common scenario has started to emerge; that is, mitochondria dysfunction triggers oxidative stress, which, in turn, leads to depleted plasmalogens and chronic inflammation. Hence, it seems reasonable to propose that a decrease in plasmalogen levels is tightly linked with these biological processes. The literature has established general roles that plasmalogens play within the cellular environment; however, more in-depth analysis of these functions is necessary as well as the dependence on their molecular species (Dean and Lodhi, 2018). This data could provide a means of diagnosis, prognosis, and/or treatment.

RESTORING PLASMALOGEN LEVELS AS A THERAPEUTIC STRATEGY

In conditions with altered lipid metabolism, a therapeutic strategy that has been considered involves the use of small molecules that could restore lipid homeostasis. Hence, the observation that plasmalogen levels are decreased in several pathophysiological conditions opens a new avenue for the development of potential therapies to these conditions, that is, plasmalogen replacement therapy (PRT). The idea behind PRT is to administer purified plasmalogens and/or plasmalogen precursors to normalize plasmalogen levels.

Administration of plasmalogen and/or its precursors has been utilized in different clinical settings to increase plasmalogen levels as well as a strategy to prevent/attenuate different pathological conditions (Das et al., 1992; Marigny et al., 2002; Brites et al., 2011; Bozelli et al., 2020b). One of the most used small molecules in PRT is AG, which is a plasmalogen precursors that enters the biosynthesis pathway in the ER after being phosphorylated in the cytosol (Synder, 1992; Braverman and Moser, 2012). For instance, AG has been shown to restore plasmalogen levels in fibroblast cells derived from ZS and RCDP patients (Brites et al., 2004). Administration of AG to a cell model of BTHS restored plasmalogen level and partly CL levels as well as improved mitochondria fitness (Bozelli et al., 2020b). In a mouse model of RCDP (*Pex7*$^{hypo/null}$) ingestion of a synthetic vinyl-ether plasmalogen restored plasmalogen levels in the plasma and increased the content at different extents in other tissues (with exception of the brain, lung, and kidney) (Fallatah et al., 2020). In addition, the treatment normalized the hyperactive behavior of *Pex7*$^{hypo/null}$ (Fallatah et al., 2020). In the brain of the Pex7 knockout mice, AG diet also did not rescue plasmalogen levels (Brites et al., 2011). However, DHA-enriched lipids have been shown to increase PE-Pls levels in the brain and, consequently, ameliorate the phenotype in a dementia mice model (Zhao et al., 2020). In a rat model of AD, ingestion of purified PE-Pls derived from *Ascidia viscera* improved cognition and learning ability (Yamashita et al., 2017). In mice models of PD, ingestion of plasmalogens or their precursors led to improved neuroprotection and immunomodulation as well as reduced neuroinflammation (Hossain et al., 2018; Nadeau et al., 2019). In ZS, PRT has been used in two patients where it has been shown to increase the levels of plasmalogen upon AG ingestion (Wilson et al., 1986). In addition, it also has been reported that memory function of AD patients with mild symptoms can be improved upon ingestion of scallop-derived plasmalogens (Fujino et al., 2017). In PD patients, ingestion of scallop-derived plasmalogens increased blood plasmalogen concentration as well as improved non-motor symptoms of PD (Mawatari et al., 2020).

The reported changes in plasmalogen levels in several diseases where chronic inflammation plays a key role opens a new avenue for their treatment. Contrary to the manipulation of the levels of other phospholipids, restoring plasmalogen levels via the use of PRT has been shown to be very successful in several disease models studied. It is crucial to understand both how plasmalogen levels are decreased as well as how their levels could be restored at the molecular level for the design of better, more potent, small molecules in clinical applications of PRT.

LOWERING PLASMALOGENS IN DISEASE: CAUSE OR EFFECT?

The steady-levels of plasmalogens are determined by their rate of biosynthesis and degradation. Alterations in plasmalogen metabolism and/or catabolism are, therefore, associated with changes in their levels. While this is a reasonable generic explanation for the alteration in plasmalogen content, the exact molecular mechanism varies with the pathophysiological condition. For instance, in peroxisome diseases plasmalogen content loss is, usually, a result of impaired biosynthesis. In ZS plasmalogen biosynthesis is deficient due to a lack of functional peroxisomes, while in RCDP the impaired biosynthesis is a consequence of mislocalization and/or absence of functional peroxisomal enzymes responsible to initiate plasmalogen biosynthesis, such as DHAP-AT and AGPS (Nagan and Zoeller, 2001; Brites et al., 2004). On the other hand, during aging as well as in degenerative (AD, PD, MS) and metabolic (BTHS, CAD) diseases, it seems that plasmalogen degradation enhancement is responsible for the lowering in plasmalogen levels. In all these conditions, mitochondria are dysfunctional, and there is an increase in the inflammatory response and oxidative stress. One way to degrade plasmalogens is via the oxidation of the enyl-ether bond, a condition that is favored upon increasing oxidative stress in the cell (Hahnel et al., 1999b; Zoeller et al., 2002). In addition, it has been reported that there is a link between oxidative stress and the activity of enzymes along the plasmalogen degradation pathways, such as cytochrome c (which acts as a plasmalogenase cleaving the enyl-ether bond) and cytosolic PLA2 (which hydrolyzes AA at the *sn*-2 position to produce eicosanoids) (Chuang et al., 2015; Jenkins et al., 2018; Kimura et al., 2018).

There is a good correlation between diseases with chronic inflammation and a lower level of plasmalogens (Wang, 1999; Spiteller, 2006). Conversely, administration of plasmalogens to individuals with these diseases reduces the extent of inflammation. Inflammation is a factor in aging, and it has been shown to play a key role in degenerative and metabolic diseases. One of the best studied examples is AD.

It has been shown in postmortem brains that there is a 60% decrease in PE-Pls relative to PE in affected brain regions of AD patients. Furthermore, this decrease in PE-Pls was specific to brain regions with histological damage characteristic of the disease and not in unaffected regions of the brain of the same individual (Ginsberg et al., 1995). This lower level of PE-Pls is, however, not limited to regions of the brain where there is morphological damage but is even seen in the levels of PE-Pls in circulation, which correlated with a characteristic AD biomarker, *i.e.,* an increased level of the protein Tau in the cerebrospinal fluid (Kling et al., 2020). The role of plasmalogens in AD has been recently reviewed (Su et al., 2019). Inflammation caused by the administration of bacterial LPS resulted in a decreased level of plasmalogen in the brain as well as the accumulation of Aβ peptides. These changes were reversed by the administration of plasmalogens (Ifuku et al., 2012). In addition to AD, other neurodegenerative diseases are associated with aging that results in the decline of plasmalogen levels caused by defects in the ability of peroxisomes to synthesize plasmalogens (Jo and Cho, 2019). Peroxisomes also contribute to the production of cytokines during inflammation (di Cara et al., 2017). Peroxisomal lipid synthesis regulates inflammation by sustaining neutrophil membrane phospholipid composition and viability (Lodhi et al., 2015). Peroxisomal alterations in the brains of patients with AD and with PD suggest that peroxisomal defects may facilitate the development of neurodegenerative disorders (Cipolla and Lodhi, 2017; Deori et al., 2018). Neurodegenerative diseases are strongly associated with oxidative stress (Wanders, 2014; Cipolla and Lodhi, 2017). Several reviews have appeared associating oxidative stress with neurodegenerative diseases (Jiang et al., 2016; Kamat et al., 2016; Puspita et al., 2017). Neuroinflammation results in the accumulation of 2-chlorohexadecane in brain lipids of endotoxin-treated mice indicating that inflammatory conditions may deplete plasmalogen levels (Üllen et al., 2010).

The series of events that results in lowering plasmalogen levels in the brain is believed to be associated with an oxidation process (Senanayake and Goodenowe, 2019). Damaged peroxisomal functions as well as higher levels of H_2O_2 potentially cause permanent plasmalogen deficiency that led to membrane changes, signaling abnormalities, neurotransmission deficits, and lowering antioxidant defenses (Braverman and Moser, 2012). Oxidative stress associated with inflammation can accelerate plasmalogen degradation by cleaving the vinyl-ether bond, further reducing the anti-inflammatory and antioxidative capacity of the tissues initiating an irrevocable vicious cycle that progresses to pathological abnormalities (Su et al., 2019). It has been proposed that cytochrome c-mediated degradation of plasmalogens due to increased oxidative stress as a potential mechanism responsible for the decrease in plasmalogens (Jenkins et al., 2018). Thus, relating oxidative stress with the loss of plasmalogens leading to disease (Jenkins et al., 2018).

Elevated levels of plasmalogen peroxides relative to plasmalogens can be detected in aging brains and in AD-affected brains providing further evidence of the significance of the maintenance of plasmalogens in the intact state of the brain (Weisser et al., 1997). Inflammation has been shown to be related to apoptosis and the generation of inflammatory caspases (Davies et al., 2021). Caspases are initiators of apoptosis and neurodegeneration (Budihardjo et al., 1999). Caspases are also associated with inflammation (Wu et al., 2009). TNFα (tumor necrosis factor α), a signaling molecule produced in inflammatory conditions, induces caspase-dependent inflammation in renal endothelial cells through a Rho- and myosin light chain kinase-dependent mechanisms. Among different caspases, caspase-3 is of particular interest because it is found to be associated with the pathologies of neurodegenerative diseases, such as AD (Su et al., 2001; D'Amelio et al., 2011). Recent studies also reported that caspase-3 is associated with the formation of amyloid-β (Aβ) by processing of amyloid precursor protein (Stone et al., 2002). Caspase-8 and caspase-3 have been implicated in microglial activation by regulating protein kinase C (Burguillos et al., 2011). Plasmalogens inhibit LPS-induced Aβ formation and microglial activation in the mouse brain cortex (Ifuku et al., 2012). Plasmalogens also suppress apoptosis in intestinal tract cells by attenuating induced inflammatory stress (Nguma et al., 2021b). Dietary PE-Pls has been shown to reduce intestinal inflammation, oxidative stress, and the expression of apoptosis-related proteins in the colon mucosa (Nguma et al., 2021a). Inflammation has also been suggested to play a role in cancer (Lan et al., 2021).

In cancer and degenerative diseases, ferroptosis (an iron-dependent, non-apoptotic cell death process) plays an important role (Stockwell et al., 2017). The increase in the levels of peroxidized intracellular lipids due to the oxidation of PUFA moieties in membrane phospholipids is responsible for triggering ferroptosis (Conrad and Pratt, 2019). Recently, it has been shown that plasmalogens, which are enriched in PUFA, can induce ferroptosis by providing PUFA for lipid peroxidation (Zou et al., 2020). Likewise, plasmalogen biosynthesis has been reported to mediate a new axis of ferroptosis, which is dependent on long-chain saturated fatty acids (Cui et al., 2021). It has been shown that the enzymes Far1 and TMEM189, which catalyze reactions in plasmalogen biosynthesis (see above), can mediate the new axis of ferroptosis (Cui et al., 2021). Both ferroptosis and plasmalogens play a role in inflammatory processes (Braverman and Moser, 2012; Sun et al., 2020). However, it is not currently understood the molecular mechanisms of the interplay between ferroptosis and plasmalogens in inflammatory processes. Future research in the field will help expand our understanding of the role of plasmalogens in inflammation.

Phagocytosis by macrophages plays an important role in controlling inflammation. Brain inflammation may be a consequence of attack by macrophages (Xiong et al., 2016). Cells deficient in PE-Pls have a reduced ability to phagocytize opsonized zymosan particles (Rubio et al., 2018). This defect can be reversed by incubating the plasmalogen deficient cells with lysoplasmalogen, which, presumably, acts as a metabolic precursor to plasmalogens. Because of the increased level of

plasmalogens the number and size of lipid domains in the membrane is increased, membrane fluidity is lowered to levels found in cells containing normal plasmalogen levels, and receptor-mediated signaling becomes more efficient.

Activation of protein kinase C delta (PKCδ) is linked to neuroinflammation. Knocking out PKCδ in mice results in resistance to inflammation, while upregulation of PKCδ in microglial cells promotes neuroinflammation (Ren et al., 2014; Gordon et al., 2016). Microglial activation is a pathological feature of many neurodegenerative diseases (Ali et al., 2019). The presence of activated microglia and reactive astrocytes that produce cytokines are associated with AD pathologies (Apelt and Schliebs, 2001; Tahara et al., 2006; Salminen et al., 2008). Plasmalogens have been shown to inhibit neuronal cell death by suppressing an intrinsic apoptotic pathway, which is characterized by the activation of caspase-9 (Hossain et al., 2013). It was also found that the systemic LPS-induced activation of microglial cells and the expression of pro-inflammatory cytokines were significantly attenuated by the administration of plasmalogens (Ifuku et al., 2012).

Toll-like receptors (TLR) plays a wide role in innate and adaptive immune responses upon stimulation by exogenous and endogenous TLR ligands. Among TLR, the TLR4 has attracted increased attention due to its ability to recruit different adaptor proteins. LPS-induced inflammatory signaling is associated with the endocytosis of TLR4. The pretreatment of cells with plasmalogens attenuated the LPS-induced signaling by inhibiting the dynamin-dependent internalization of TLR4. Knockdown of the plasmalogen synthesizing enzyme, DHAP-AT, by lentiviral vectors encoding short hairpin-RNA against DHAP-AT resulted in the increased activation of caspases and the endocytosis of TLR4, which was reversed by the ingestion of plasmalogens (Ali et al., 2019). The LPS-TLR4 complex initiates the TLR4 endocytosis, which is believed to play a major role in regulating inflammatory signals to induce cytokine expression by activating the Toll/interleukin-1 receptor domain-containing adaptor protein and the MyD88 adaptor proteins, as well as Toll/IL-1R domain-containing adaptor inducing type I interferons-mediated pathways in mouse macrophages and Ba/F3 cells (Akira and Takeda, 2004; Kagan et al., 2008; Wong et al., 2009). The internalization of TLR4 has been reported to be mediated by clathrin-dependent endocytosis in HEK 293 cells, lipid domain-mediated endocytosis in CHO cells, and both clathrin-dependent and lipid domain-mediated endocytosis in cortical astrocytes (Shuto et al., 2005; Husebye et al., 2006; Pascual-Lucas et al., 2014).

Inflammation is accompanied by many changes as outlined above. These include mitochondria dysfunction, oxidative stress, apoptosis and the increased expression of caspases, phagocytosis by macrophages, activation of PKCδ, stimulation of microglia with the generation of inflammatory cytokines, and TLR signaling. Many of these processes are associated with pathologies caused by inflammation and are reversed by administering plasmalogens and/or plasmalogen precursors. Plasmalogen levels are reduced in diseases that affect these processes. More complicated, however, is determining which of the processes cause the dysfunctions and which are consequences of the inflammatory process itself. It can be concluded that plasmalogens are at least protective against cell or tissue damage caused by inflammation. This is supported by the finding that in many examples of pathologies caused by inflammation, there is a loss of plasmalogens. Furthermore, administration of plasmalogens or plasmalogen precursors can prevent tissue damage caused by inflammation. A mechanism can be proposed to explain the protective effect of plasmalogens. Inflammation is often accompanied by the production of ROS causing oxidative damage to tissues. Plasmalogens are protective against oxidative damage because of their enyl-ether linkage that is highly susceptible to oxidation by ROS, thus preventing ROS from attacking at other sites. Deciding if the loss of plasmalogens is a cause or a consequence is to some extent a matter of definitions and will vary from one disease to another. In some cases, the loss of plasmalogens is the primary defect, such as inflammation related to aging in which the ability to synthesize plasmalogens is decreased. However, in other cases, such as the production of inflammatory cytokines, other processes may occur first. Nevertheless, even in these cases, plasmalogens play a protective role and can prevent inflammation. In that sense, even when the loss of plasmalogens is not the first event, their presence or absence can determine the course of inflammation.

AUTHOR CONTRIBUTIONS

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REVIEW
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Studying Lipid-Related Pathophysiology Using the Yeast Model

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Saccharomyces cerevisiae, commonly known as baker's yeast, is one of the most comprehensively studied model organisms in science. Yeast has been used to study a wide variety of human diseases, and the yeast model system has proved to be an especially amenable tool for the study of lipids and lipid-related pathophysiologies, a topic that has gained considerable attention in recent years. This review focuses on how yeast has contributed to our understanding of the mitochondrial phospholipid cardiolipin (CL) and its role in Barth syndrome (BTHS), a genetic disorder characterized by partial or complete loss of function of the CL remodeling enzyme tafazzin. Defective tafazzin causes perturbation of CL metabolism, resulting in many downstream cellular consequences and clinical pathologies that are discussed herein. The influence of yeast research in the lipid-related pathophysiologies of Alzheimer's and Parkinson's diseases is also summarized.

Keywords: *Saccharomyces cerevisiae*, lipids, cardiolipin, Barth syndrome, pathophysiology, tafazzin

INTRODUCTION

Saccharomyces cerevisiae is a powerful model system used to study biological processes and human diseases. In addition to investigating the pathophysiology of diseases, yeast is also used as a model for developing and testing potential treatments. An excellent example of the power of the yeast model is the use of yeast cardiolipin (CL) synthesis mutants to understand the metabolic abnormalities in Barth syndrome (BTHS), a rare genetic disorder caused by mutations in the CL-remodeling enzyme tafazzin (Bione et al., 1996; Vreken et al., 2000). CL is a unique phospholipid localized primarily in the inner mitochondrial membrane (IMM). Yeast CL mutants have been pivotal in elucidating the role of this lipid in bioenergetics (Paradies et al., 2014; Ren et al., 2014; Raja et al., 2017a), mitochondrial metabolism (Houtkooper and Vaz, 2008; Raja et al., 2019), and programmed cell death (Manon, 2004; Eisenberg and Buttner, 2014; Lou et al., 2018b) among other cellular functions. This review aims to demonstrate how the yeast model has led the way for BTHS studies and contributed to recent advances in our understanding of other human diseases.

THE MITOCHONDRIAL DISORDER BARTH SYNDROME

History

In 1983, physician Peter Barth reported the first description of the disorder that bears his name, describing it as "an X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle, and neutrophil leukocytes" (Barth et al., 1983). It was not until 1996 that the cause of BTHS was linked to mutations in the TAZ gene (Bione et al., 1996). Vreken et al. (2000) discovered that TAZ mutations lead to a profound defect in CL remodeling. During 2003–2004, the first research model for studying BTHS – yeast *taz1*Δ – was constructed (Vaz et al., 2003; Gu et al., 2004; Ma et al., 2004).

Since the emergence of the yeast *taz1*Δ model, numerous other BTHS model systems have been developed in different organisms through targeted disruption of the tafazzin gene. In 2006, the first whole-animal models were generated in fruit flies (Xu et al., 2006) and zebrafish (Khuchua et al., 2006). Subsequent efforts in 2011–2012 led to the development of the first mouse models of BTHS, facilitating tissue-specific studies of tafazzin deficiency in organs such as the heart and skeletal muscle (Acehan et al., 2011; Soustek et al., 2011; Phoon et al., 2012). More recently, BTHS cell models have been developed in immortalized mammalian cell lines, including mouse C2C12 myoblasts (Lou et al., 2018a) and human HEK293 kidney cells, which provide the added experimental benefit of having isogenic control cells.

Epidemiology

As an X-linked recessive disorder, BTHS is predominantly diagnosed in male patients. It has been suggested that females carrying single-allele TAZ mutations exhibit a skewed pattern of X chromosome inactivation, resulting in a normal clinical presentation (Orstavik et al., 1998). BTHS is exceptionally rare, with only 151 living patients identified worldwide in 2012 (Clarke et al., 2013). Approximately 10 new BTHS cases are diagnosed each year in the United States, which translates to a prevalence of 1:300,000–400,000 live births (Clarke et al., 2013). However, it is likely that BTHS is underdiagnosed due to premature infant mortality and misdiagnosis of children presenting with cardiomyopathies.

Genetic Basis of BTHS

BTHS results from mutations in the Tafazzin (TAZ) gene (originally referred to as G4.5). TAZ is a mitochondrial transacylase that re-acylates monolysocardiolipin (MLCL) by adding predominantly unsaturated acyl chains (Barth et al., 2004; Schlame and Xu, 2020). It is the primary enzyme that conducts this function, and as such, TAZ mutations result in elevated MLCL levels, decreased overall CL, and increased saturated fatty acid content in CL (Vreken et al., 2000; Schlame et al., 2003; Gu et al., 2004; Valianpour et al., 2005).

TAZ is located on chromosome Xq28 and contains 11 exons (Bolhuis et al., 1991; Bione et al., 1996). Although multiple mRNA splice variants exist, the only detectable form of TAZ protein in human fibroblasts lacks exon 5 (Lu et al., 2016). However, BTHS-associated mutations have been identified in all TAZ exons, including exon 5, suggesting that full-length TAZ protein is also physiologically relevant *in vivo* (Cantlay et al., 1999; Gonzalez, 2005). To date, more than 180 pathogenic TAZ gene mutations have been identified, ranging from single nucleotide polymorphisms to whole-gene deletion (Singh et al., 2009). A major enigma in BTHS research is the apparent discrepancy between specific TAZ mutations and the clinical phenotypes they result in. For example, individuals sharing an identical mutation can have contrasting clinical presentations that range from severe heart failure and hypotonia to being nearly asymptomatic (Ronvelia et al., 2012).

Pathology

Cardiomyopathy

BTHS and its clinical manifestations have been previously discussed in other reviews (Raja et al., 2017b; Ghosh et al., 2019; Taylor et al., 2021; Zegallai and Hatch, 2021). Cardiomyopathy is the major clinical manifestation of BTHS, and all identified patients have developed cardiomyopathy at some point in their lives. Dilated cardiomyopathy is the most common form in BTHS patients and is often associated with hypertrophy, left-ventricular noncompaction, arrhythmia, conduction defects, and endocardial fibroelastosis (a heart disorder in children characterized by diffuse thickening of the endocardium; Brady et al., 2006; Raja et al., 2017b). Hypertrophic and dilated phases can be recurring over a patient's lifetime (Ferreira et al., 2014). Cardiomyopathy increases the risk of arrhythmia, conduction defects, and congestive heart failure and may lead to sudden cardiac death (Spencer et al., 2006; Yen et al., 2008). In addition, cardiomyopathy in BTHS can be diagnosed late or misdiagnosed, leading to cardiac failure (Spencer et al., 2006; Mangat et al., 2007).

Fortunately, BTHS patient outcomes have improved significantly in the past two decades. Patients born after the year 2000 have a 5-year survival rate of 70% compared to 22% for those born before 2000 (Rigaud et al., 2013). This change suggests that early identification and management of heart dysfunction can significantly improve survival rates for BTHS patients. Although the molecular mechanisms are not known, studies have suggested that several factors, including mitochondrial dysfunction, defective mitochondrial protein import, autophagy, lipid storage myopathy, reduced glucose oxidation, and deficient muscle development, can all contribute to the onset of cardiomyopathy in BTHS (Shen et al., 2015; Greenwell et al., 2021).

Skeletal Myopathy

Skeletal myopathy is widely observed in BTHS, and patients often present with a combination of muscle weakness and wasting, delayed gross motor development, pre-pubescent growth delay, and/or hypotonia (Spencer et al., 2006, 2011; Bittel et al., 2018). In BTHS, this condition is usually non-progressive and mainly affects proximal skeletal muscle (Clarke et al., 2013; Ferreira et al., 2014; Mazar et al., 2019) Growth delay usually

regresses over time and is often followed by accelerated growth during mid and late puberty (Reynolds et al., 2015). Similar to cardiomyopathy, the mechanism underlying development of skeletal myopathy in BTHS is not well understood.

Exercise Intolerance

In a self-assessment, BTHS patients identified exercise intolerance as the clinical feature that most negatively impacts their daily life. The reduced capacity for physical activity in BTHS is thought to result from both cardiac impairment (i.e., overall endurance) and diminished skeletal muscle oxygen utilization (Spencer et al., 2011). Not only does exercise intolerance pose a physical limitation on the ability of patients to independently perform everyday tasks, but it also serves as a psychosocial barrier that likely contributes to reports of lower quality of life ratings from BTHS patients relative to their peers (Storch et al., 2009; Mazar et al., 2019).

Neutropenia

The severity of neutropenia in BTHS ranges from mild (benign, transient neutropenia) to severe (congenital neutropenia affecting multiple organs; Folsi et al., 2014). Bacterial or viral infections resulting from neutropenia can cause significant complications throughout a patient's life, including the risk of death by sepsis in the most extreme cases. According to the first report by Barth et al. (1983) three out of his seven patients died prematurely due to septicemia. The molecular mechanisms underlying the development of neutropenia in BTHS are still largely unknown.

Other Clinical Manifestations

While the above pathologies constitute the core clinical features of BTHS, a range of other clinical manifestations have been reported. Some patients develop facial dysmorphism characterized by a tall, broad forehead, round face, full cheeks, and large ears (Ferreira et al., 2014). Additionally, dysmorphism of the feet resulting in talipes equinovarus (clubfoot) has been reported at a higher incidence in BTHS patients (Ades et al., 1993; Spencer et al., 2011). Cognitive and neurological phenotypes have also been described. For example, a study of 15 adolescent BTHS patients found reduced abilities in mathematics and visual spatial tasks (Mazzocco et al., 2007), and a recent study also identified deficiencies in balance and motion reaction time in a group of 33 BTHS patients relative to age-matched controls (Hornby et al., 2019). Other phenotypes include a strong gag reflex (Reynolds et al., 2012), sideways curvature of the spine (Roberts et al., 2012), and increased male fetal loss, stillbirth, and neonatal death (Marziliano et al., 2007; Steward et al., 2010).

CARDIOLIPIN AND BARTH SYNDROME

The Cardiolipin Biosynthetic Pathway

CL Synthesis

In order to comprehend how the yeast model has furthered our understanding of BTHS pathophysiology, it is necessary to understand the details of the CL biosynthetic pathway, many of which were first discovered in yeast. CL is a dimeric phospholipid in which two phosphatidyl groups are connected by a central glycerol molecule (Lecocq and Ballou, 1964). It is synthesized and localized predominantly in the inner leaflet of the IMM (Hostetler and van den Bosch, 1972; Krebs et al., 1979; Schlame and Haldar, 1993; Gebert et al., 2009; Joshi et al., 2009; Schlame and Ren, 2009; Sparagna and Lesnefsky, 2009; Osman et al., 2011).

CL biosynthesis is a four-step process that utilizes phosphatidic acid (PA) as substrate (**Figure 1**). PA is synthesized in both the endoplasmic reticulum (ER) and the outer leaflet of the outer mitochondrial membrane (OMM; Chakraborty et al., 1999). In both cases, the translocation of PA from the OMM to the inner leaflet of the IMM *via* the yeast Ups1/Mdm35 lipid transport complex (PRELID1/TRIAP1 in humans) is essential for CL synthesis (Potting et al., 2010; Tamura et al., 2010; Connerth et al., 2012). In the first step of the biosynthetic pathway, PA in the IMM is converted into cytidine diphosphate diacylglycerol (CDP-DAG) by Tam41 in yeast (TAM41 in humans; Blunsom et al., 2018). Next, the yeast mitochondrial enzyme Pgs1 or its human homolog, PGS1, converts CDP-DAG into phosphatidylglycerol phosphate (PGP) by transferring a phosphatidyl group from CDP-DAG onto glycerol-3-phosphate (G3P; Chang et al., 1998a). PGP is then dephosphorylated to phosphatidylglycerol (PG) by the yeast enzyme Gep4 (PTPMT1 in humans; Osman et al., 2010; Zhang et al., 2011). Finally, a phosphatidyl group from CDP-DAG is transferred to PG by the yeast enzyme Crd1 (hCLS1 in humans) to synthesize nascent CL (Schlame and Greenberg, 1997; Chang et al., 1998b).

CL Remodeling

Newly synthesized CL undergoes a unique remodeling process in which saturated acyl chains are replaced with unsaturated acyl chains through several cycles of deacylation and reacylation. The yeast enzyme Cld1 is responsible for the first step in CL remodeling by deacylating CL to form MLCL (Beranek et al., 2009). In the second step, MLCL is reacylated by tafazzin (Taz1 in yeast or TAZ in humans) to form primarily unsaturated CL (**Figure 1**; Xu et al., 2006).

In mammals, multiple enzymes are capable of deacylating CL, though none of them are direct homologs of the yeast enzyme Cld1. These include iPLA2γ, iPLA2β, cPLA2, and sPLA2 (Buckland et al., 1998; Mancuso et al., 2007; Dennis et al., 2011; Hsu et al., 2013). In addition to TAZ, MLCLAT1 and ALCAT1 can also catalyze the reacylation of MLCL in mammalian cells, while there are no direct homologs of these two enzymes in yeast (Ma et al., 1999; Cao et al., 2004; Li et al., 2010; Mejia et al., 2018). As a result of diminished tafazzin function, BTHS is characterized by an increase in MLCL levels, along with a decrease in unsaturated and total CL (Jiang et al., 1997; Xu et al., 2006; Lou et al., 2018a; Wang et al., 2020). The fact that these phenotypes arise despite the presence of MLCLAT1 and ALCAT1 suggests that TAZ is the primary enzyme responsible for reacylation of MLCL under physiological conditions (Saric et al., 2015).

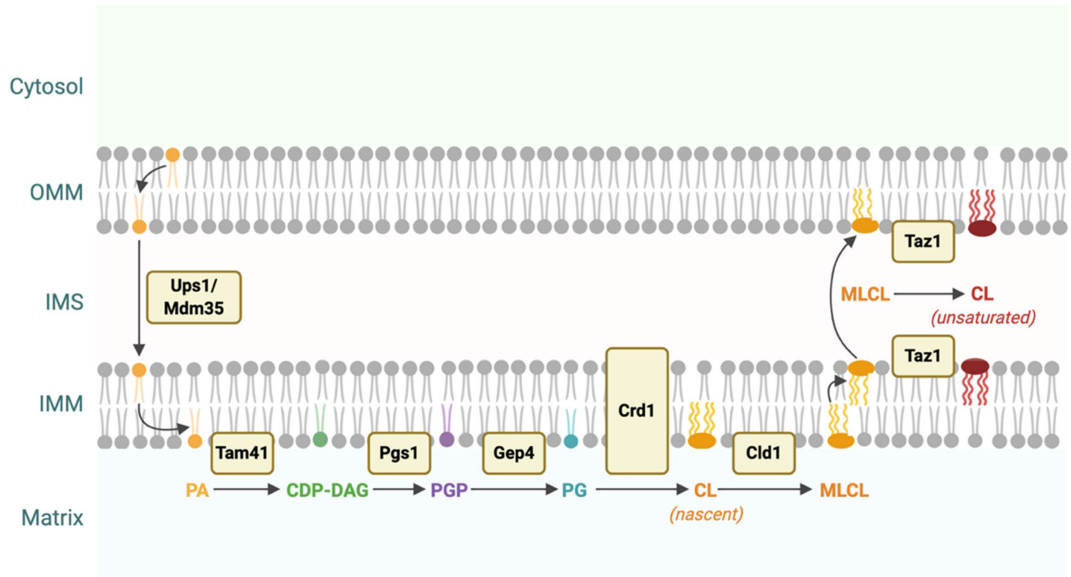

FIGURE 1 | Cardiolipin (CL) biosynthesis in yeast. CL is synthesized from phosphatidic acid (PA) through a four-step process. PA is translocated from the outer mitochondrial membrane (OMM) to the IMM *via* the Ups1/Mdm35 protein complex (Potting et al., 2010; Tamura et al., 2010; Connerth et al., 2012). PA is converted into cytidine diphosphate diacylglycerol (CDP-DAG), phosphatidylglycerol phosphate (PGP), and then phosphatidylglycerol (PG) by Tam41, Pgs1, and Gep4, respectively (Chang et al., 1998a; Osman et al., 2010; Blunsom et al., 2018). Finally, PG is converted to nascent CL by the enzymatic activity of Crd1. Nascent CL, which contains predominantly saturated acyl chains, is remodeled through several cycles of deacylation and reacylation catalyzed by Cld1 and Taz1 to form primarily unsaturated CL (Xu et al., 2006; Beranek et al., 2009). This figure was created with BioRender.com.

STUDYING CL-RELATED PATHOLOGIES USING YEAST MUTANTS

Yeast is an excellent model for studying genetic disorders. In 1996, *S. cerevisiae* was the first eukaryote for which a full genome sequence was assembled (Goffeau et al., 1996; Giaever and Nislow, 2014). Subsequently, researchers generated a deletion collection containing representative mutant strains for each nonessential gene in the yeast genome (~4,800 genes; Giaever and Nislow, 2014). Using these tools, homologs of over 23% of all human genes have been identified and studied in yeast (Kachroo et al., 2015). One of the most striking discoveries from these endeavors was that many genes encoding lipid pathways are conserved from yeast to humans, thus making yeast a useful model to interrogate human diseases of lipid metabolism. The ability of yeast to grow both as haploid or diploid cells allows for the construction of double mutants by crossing single mutants, inducing sporulation, and screening the resulting haploids. In addition to being genetically tractable, yeast cells are nonpathogenic, have a short generation time, and are easy and inexpensive to culture in the laboratory.

The CL biosynthetic pathway is conserved from yeast to humans, and yeast mutants have been constructed for each step of this pathway. Among these, the *crd1*Δ mutant (Jiang et al., 1997; Tuller et al., 1998; Chang et al., 1998b), which lacks CL synthase and cannot synthesize CL, and the *taz1*Δ mutant (Vaz et al., 2003; Gu et al., 2004; Ma et al., 2004), lacking tafazzin, have been pivotal in elucidating the cellular roles of CL and understanding BTHS pathology. Yeast mutants have also been generated to recapitulate and test the functional significance of human TAZ mutations identified in BTHS patients (Claypool et al., 2006, 2011). The following sections detail pioneering discoveries relevant to BTHS pathophysiology that were first made using yeast mutants.

CL and Bioenergetics

CL comprises 15–20% of the phospholipid content in the IMM where it plays a pivotal role in energy metabolism (Pennington et al., 2019). The mitochondrial III_2IV_2 supercomplex forms the terminal part of the electron transport chain and is essential for maintaining mitochondrial membrane potential and ATP synthesis (Schagger and Pfeiffer, 2000). In *crd1*Δ yeast, the III_2IV_2 supercomplex is less stable than in wildtype cells, suggesting that CL is critical for mitochondrial homeostasis and bioenergetics (Pfeiffer et al., 2003; Mileykovskaya et al., 2005; Zhang et al., 2005; Claypool et al., 2008; Bottinger et al., 2012; Bazan et al., 2013; Peyta et al., 2016; Petit et al., 2020). A deficiency in CL remodeling is also associated with reduced bioenergetics (Brandner et al., 2005; Li et al., 2007). Using *taz1*Δ yeast, Brandner et al. (2005) demonstrated that the absence of tafazzin results in increased dissociation of the III_2IV_2 supercomplex, causing the release of a complex IV monomer (Brandner et al., 2005; Claypool et al., 2008).

The initial yeast studies implicating CL and CL remodeling in bioenergetics were subsequently corroborated using mammalian BTHS models. Gonzalvez et al. (2013) and McKenzie et al. (2006) reported decreased respiratory supercomplex formation and stability in lymphoblast cells isolated from two

BTHS patients relative to non-BTHS control cells. More recently, Petit et al. (2020) showed that shRNA-mediated Taz knockdown in HeLa cells results in decreased ATP synthase activity and overall ATP level, with a concomitant decrease in maximal respiratory capacity and an increase in basal oxygen consumption. Similarly, Dudek et al. (2013, 2016) showed reduced respiratory complex formation in cardiac tissue isolated from the BTHS mouse model and increased basal oxygen consumption coupled with decreased maximal respiratory capacity in induced pluripotent stem cells (iPSCs) derived from BTHS patients. Ma et al. (2004) reported a decrease in basal respiration in *taz1*Δ yeast, a study corroborated by Lou et al. (2018a) in C2C12 TAZ KO (Ma et al., 2004). The increased basal respiration observed in iPSC-derived cardiomyocytes could be due to increased F1F0 ATP synthase oxygen consumption and proton leak. The authors further showed that the aberrant respiration in iPSCs could be attributed to reduced respiratory complex stability, reiterating what was previously concluded from yeast. These findings underscore the power of the yeast model for interrogating the relationship between CL deficiency and the bioenergetic defects that characterize BTHS.

CL and Iron Homeostasis

Iron–sulfur clusters (ISCs) are molecular assemblages of iron and sulfur atoms that act as co-factors in many cellular processes, including electron transfer within the electron transport chain and enzymatic conversion of substrate within the TCA cycle (Paul et al., 2017; Braymer et al., 2021). ISCs can exist in many configurations based on the number of iron and sulfur atoms involved (e.g., 2Fe-2S, 3Fe-4S, and 4Fe-4S). ISC biogenesis occurs in three steps within mitochondria (Lill and Freibert, 2020). The first step involves donation of sulfur from the NFS1-ISD11-ACP1 subcomplex and transfer of imported iron to the ISCU2 scaffold protein by frataxin (FXN). This forms an initial 2Fe-2S cluster. In the second step, chaperone proteins (HSC20, HSPA9, and GRPE1) bind to the 2Fe-2S cluster and transfer it first to the monothiol glutaredoxin GLRX5 and subsequently to mitochondrial ISC recipient proteins. The third step involves conversion of 2Fe-2S clusters into 4Fe-4S clusters and their delivery to recipient apoproteins (Lill and Freibert, 2020; Maio et al., 2020).

The first indication of a relationship between CL and ISC homeostasis was described in yeast. Using *crd1*Δ yeast, Patil et al. (2013) demonstrated that CL deficiency results in elevated mitochondrial iron and increased sensitivity to ROS and exogenously supplied iron sulfate. These phenotypes are associated with defective ISC biogenesis, and the authors subsequently showed that *crd1*Δ cells exhibit decreased enzymatic activity of the ISC-requiring enzymes ubiquinol-cytochrome *c* oxidoreductase, succinate dehydrogenase, aconitase, isopropylmalate isomerase, and sulfite reductase. Additionally, deletion of the ISC biosynthetic gene ISU1 in the *crd1*Δ background resulted in a synthetically sick phenotype, further supporting a role for CL in iron homeostasis (Patil et al., 2013).

These initial findings from yeast were subsequently validated in mammalian cells using the TAZ-KO C2C12 BTHS cell model. Similar to what was observed in yeast, Li et al. (2020) demonstrated that the activities of the ISC-requiring enzymes aconitase, NADH dehydrogenase, succinate dehydrogenase, and ubiquinol-cytochrome *c* reductase were all decreased by ~50% in TAZ-KO cells while their respective protein levels remained unchanged. TAZ-KO cells also showed increased mitochondrial iron content and elevated sensitivity to ROS and iron supplementation, mirroring the findings from yeast. This study went on to further corroborate the role of CL in ISC biogenesis by showing that the mature form of the ISC biosynthetic protein FXN is reduced in TAZ-KO cells (Li et al., 2020).

CL and Energy Metabolism

Raja et al. (2017a) provided the first evidence of a link between CL and energy metabolism by showing that *crd1*Δ exhibits decreased synthesis of acetyl-CoA, and that deletion of *CRD1* is synthetically lethal in pyruvate dehydrogenase (PDH) mutants. Acetyl-CoA is a primary substrate utilized by the tricarboxylic acid (TCA) cycle to fuel intermediary metabolism, and under respiratory conditions it is synthesized predominantly in mitochondria through the enzymatic conversion of pyruvate by PDH (Guest et al., 1989; Raja et al., 2017a). Synthetic lethality between *crd1*Δ and PDH complex mutants suggests that CL plays a role in promoting acetyl-CoA synthesis and TCA cycle function. Interestingly, PDH complex mRNA and protein levels are increased in *crd1*Δ, but net activity of PDH is not altered (Raja et al., 2017a). This suggests that CL is required for optimal PDH function, and that in the absence of CL, upregulation of PDH cannot compensate for diminished acetyl-CoA synthesis.

Building on the findings in yeast, Li et al. (2019) found that TAZ-KO mouse C2C12 cells also show reduced carbon flux to acetyl-CoA, and this is linked to a reduction in PDH activity. PDH is regulated through phosphorylation, and the authors showed that both the inhibitory phosphorylation and enzymatic activity in mitochondrial extracts are rescued by the addition of exogenous CL. Although production of acetyl-CoA through the activity of PDH serves as an important input for the TCA cycle, the cycle itself is comprised of many steps that are each catalyzed by distinct enzymes, some of which require Fe-S cofactors for optimal activity. Li et al. (2019, 2020) identified a second way in which CL deficiency negatively impacts TCA cycle function by showing that activity of the Fe-S-requiring TCA cycle enzymes succinate dehydrogenase and aconitase are also reduced in TAZ-KO cells.

CL Enhances the Stability of the Mitochondrial Calcium Uniporter

The mitochondrial calcium uniporter (MCU) is a holo-complex protein consisting of pore-forming subunit MCU, transmembrane subunit EMRE, and regulatory subunits MICU1, MICU2, and MCUb (Baughman et al., 2011; De Stefani et al., 2011; Sancak et al., 2013). MCU transports calcium from the cytosol into the mitochondria where it serves as a signal for regulating ATP synthesis and, when in excess, activating the apoptotic pathway (Carraro et al., 2020). MCU is localized in the IMM

where CL is enriched, suggesting that its function could be influenced by CL (Ghosh et al., 2020).

The first evidence for CL playing a role in MCU function came from a recent study in yeast, where Ghosh et al. (2020) reported that MCU levels are decreased by 50% in CL-deficient *crd1*Δ cells. In this study, MCU was heterologously expressed under a strong promoter. Therefore, the authors argue that CL influences MCU stability but not expression, which is supported by the finding that the MCU turnover rate is substantially higher in *crd1*Δ compared to wildtype cells. As a functional consequence, mitochondrial uptake of calcium is also decreased in *crd1*Δ mutants. The authors further validated these yeast findings in both the TAZ-KO C2C12 mouse model and in BTHS patient-derived lymphoblasts and heart tissue. In a similar study, Ghosh et al. (2021) showed that only the MICU1 component of the mitochondrial calcium uniporter complex is decreased in *crd1*Δ cells. In this study, human MICU1 and Mitochondrial Calcium Uniporter Regulator 1 (MCUR1) proteins were expressed in both wildtype and *crd1*Δ yeast cells. The authors showed that MICU1 levels were 50% lower in *crd1*Δ cells compared to the wildtype, while MCUR1 levels were unaltered (Ghosh et al., 2021). This work serves as yet another example of how yeast research has led to pioneering discoveries regarding CL function and its relationship to BTHS.

USING YEAST TO STUDY OTHER LIPID-RELATED DISEASES

The yeast model has proven to be indispensable not only in the study of CL-related pathologies, but in other lipid-related pathologies as well. In addition, the genetic tractability, rapid doubling time, and low cost often make the use of yeast preferable to other models. When modeling human diseases in yeast there are two general approaches. The first method is referred to as an orthologous approach, in which a yeast ortholog of a human gene is modified to have the same mutation as in the human disease (e.g., the *crd1*Δ and *taz1*Δ strains used to study BTHS). Using this approach researchers can identify interactions between other genes, proteins, and molecules that may interact with the aberrant disease pathway. However, this is not always possible, as the discrepancy in genome size between yeast and humans means that not all human genes have a yeast homolog. The second method, referred to as humanization, involves expressing a human disease gene in yeast. Although some tissue-wide aspects of human diseases are difficult to model in the unicellular yeast system, cellular phenotypes can often be recapitulated and studied in yeast cells. The following sections detail ways in which yeast have been used to study the lipid-related pathophysiology of two prevalent human neurodegenerative diseases, Alzheimer's and Parkinson's.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disease in the world and is characterized by the extracellular accumulation of amyloid-β (Ab) peptide in senile plaques and the intracellular accumulation of neurofibrillary tangles (Masters et al., 1985; Alzheimer et al., 1995; Scheltens et al., 2016). These aggregations lead to neurodegeneration, with the main clinical manifestation being severe memory impairment and memory loss that often results in chronic dementia (Masliah et al., 1989; Bancher et al., 1997; Scheltens et al., 2016). While the molecular phenotypes of AD have been well cataloged, the exact pathophysiology of the disease is not well understood.

In order to gain insight into the pathophysiology of AD, Nair et al. (2014) conducted a genome-wide screen to identify cellular processes that influence Ab aggregation in yeast. In this study, the authors compared mutants from the yeast deletion collection expressing a GFP-tagged Ab construct (Ab-GFP) with wild-type cells to identify genes that influence the size or localization of Ab aggregations. Out of ~4,600 mutants tested, the screen identified 110 relevant genes corresponding predominantly to four major cellular processes, including phospholipid metabolism, gene expression, chromatin remodeling, and mitochondrial function. This study has been foundational in guiding subsequent AD research efforts.

In particular, dysregulation of lipid metabolism has been increasingly recognized as a contributing factor to AD pathophysiology, and the yeast model has been indispensable for exploring this link (Kao et al., 2020). One example of the link between lipid metabolism and AD pathology involves a neurotoxic species of phosphatidylcholine, referred to as PC(*O*-16:0/2:0), that is elevated in human AD tissue (Ryan et al., 2009). Kennedy et al. (2016) used yeast to better understand the role of this lipid species in AD pathology by combining gene expression profiling with a genome-wide chemogenomic screen. They found that elevated PC(*O*-16:0/2:0) causes an accumulation of ceramide that ultimately results in increased reactive oxygen species (ROS) production and mitochondrial dysfunction, cellular phenotypes that are commonly seen in AD patient cells and AD cell models (Agrawal and Jha, 2020).

Another example of the link between lipid metabolism and AD relates to tau protein phosphorylation. Tau hyperphosphorylation has been implicated as a major part of AD neurodegeneration, but the mechanism of hyperphosphorylation is not well understood (Simic et al., 2016). Using yeast, Randez-Gil et al. (2020) identified a potential mechanism wherein dysregulation of inositol phosphate signaling leads to defective sphingolipid production and a resultant increase in tau protein hyperphosphorylation. Although sphingolipid metabolism had been previously linked to neurodegenerative diseases (Alaamery et al., 2021), this was one of the first studies to suggest a role for SLs in tau hyperphosphorylation.

The previous studies directly link lipid metabolism to AD, but other aspects of lipid homeostasis have also been shown to contribute to AD pathology. Apolipoprotein E (APOE) is a lipid transporter that transports lipids between cells and tissues (Huang and Mahley, 2014). APOE is polyallelic, and the *E4* allele (APOE4) is a known risk factor for AD (Farrer et al., 1997). It is well characterized that APOE4 plays a role in AD, but the mechanism by which it contributes to AD is largely unknown. Using genome-wide screens and lipidomic analysis in yeast, Sienski et al. (2021) determined that APOE4

is responsible for altered fatty acid (FA) metabolism which is commonly seen in AD cells. They found that when human APOE4 is expressed in yeast there is an increase in the degree of unsaturation in FAs accompanied by the accumulation of lipid droplets. Furthermore, they also demonstrated that choline supplementation ameliorates the aberrant FA metabolism (Sienski et al., 2021). When choline was supplemented, synthesis of the membrane phospholipid phosphatidylcholine was stimulated. This abolished APOE4 lipid-related defects and suggests an important role for phosphatidylcholine in the pathophysiology of AD. This study demonstrated not only a novel role of APOE4 in AD, but also identified a key modulator of this process, choline homeostasis.

Yeast has proved to be a powerful tool for studying lipid-related pathology in AD. By combining the genetic tractability of yeast with high-throughput screening techniques, researchers have uncovered novel mechanisms into the influence of lipid homeostasis on Ab aggregation, ROS production, tau hyperphosphorylation, and FA metabolism.

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease world-wide. PD is characterized by degeneration of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta region of the brain and the presence of intraneuronal α-synuclein (aS) inclusions known as Lewy bodies (Antony et al., 2013; Leao et al., 2015; Sveinbjornsdottir, 2016). Degradation of dopaminergic neurons in this region of the brain decreases dopamine release in the striatum, altering motor control in afflicted individuals (Antony et al., 2013; Leao et al., 2015; Sveinbjornsdottir, 2016). Common motor symptoms include bradykinesia, rigidity, tremors, and postural instability, and these presentations are often accompanied by depression, anosmia, dementia, and sleep disorders (Moustafa et al., 2016; Pfeiffer, 2016). The molecular hallmarks of PD are well characterized, but just as in AD, the exact cause of PD is not known.

One of the major molecular hallmarks of PD is the aggregation of aS, a protein commonly expressed in neurons and enriched in presynaptic terminals (Maroteaux et al., 1988; Taguchi et al., 2016). It is well known that aS aggregation has a central role in PD pathogenesis, but the mechanism by which aS aggregates form is not known. Wang et al. (2014) used the yeast model to test a potential mechanism for aS aggregation and found that phosphatidylethanolamine (PE) deficiency in yeast cells causes ER stress, vesicle defects, aS aggregation, and cells death. Subsequently, the authors demonstrated that the effects of PE deficiency can be mitigated by ethanolamine supplementation (Wang et al., 2014). This study highlights the importance of lipid homeostasis on aS aggregation.

A study by Fanning et al. (2019) sought to further interrogate the relationship between aS aggregation, lipid homeostasis, and cellular toxicity. The authors performed lipidomic profiling in yeast displaying aS aggregation to monitor changes in specific lipid classes. They determined that dysfunctional lipid homeostasis, induced by aS aggregation, leads to cytotoxicity due to the accumulation of oleic acid (OA) and diglycerides (DG) in lipid droplets of aS-expressing yeast (Fanning et al., 2019). Of particular interest from a therapeutic standpoint, they found that either preventing the conversion of triglycerides (TG) to DG or inhibiting stearoyl-CoA desaturase (SCD; the rate-limiting enzyme in the production of OA) ameliorates aS aggregation and its associated cytotoxicity.

Yeast is not only an excellent model for identifying novel pathophysiological mechanisms but also for probing and testing chemical and genetic modifiers of a disease (Griffioen et al., 2006; Williams et al., 2007; Su et al., 2010). For example, Soste et al. (2019) conducted a screen for genetic modifiers of aS aggregation and identified 33 genes that modulate aS aggregation and cytotoxicity. One of these modifiers, Pah1, is an enzyme that converts PA to DG in yeast. In accordance with the findings of Fanning et al. (2019), Soste et al. (2019) found that decreasing DG levels through the inhibition of Pah1 ameliorates aS aggregation and cytotoxicity. This suggests a key role for DG and lipid droplet homeostasis in aS aggregation and cytotoxicity in PD.

Collectively, these studies demonstrate the power of the yeast system for investigating the lipid-related pathophysiology of two prominent neurodegenerative disorders. The unicellular nature of yeast makes it particularly straightforward to track biochemical changes in molecules such as lipids.

CONCLUSION

This review outlines some of the major contributions the yeast model has made to our understanding of the lipid-related pathologies observed in BTHS, AD, and PD. Lipid homeostasis has been shown to play key roles in other diseases, including cancer (Snaebjornsson et al., 2020), heart disease (Poss et al., 2020; Richardson et al., 2020), diabetes mellitus (Katsiki et al., 2017; Bjornstad and Eckel, 2018), and other chronic conditions (Leuti et al., 2020). The multifaceted roles of lipids in human disease are only beginning to be uncovered, and as technologies such as mass spectrometry continue to evolve, the yeast model system promises to continue facilitating progress in this rapidly growing field (Zullig and Kofeler, 2021).

AUTHOR CONTRIBUTIONS

TR-E, CO, MS, LV, and MG all contributed to the conception, design, and writing of the manuscript. AL contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Phospholipases and Reactive Oxygen Species Derived Lipid Biomarkers in Healthy and Diseased Humans and Animals – A Focus on Lysophosphatidylcholine

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Phospholipids (PL) are converted into lipid biomarkers by the action of phospholipases and reactive oxygen species (ROS), which are activated or released under certain physiological and pathophysiological conditions. Therefore, the *in vivo* concentration of such lipid biomarkers [e.g., lysophospholipids (LPLs)] is altered in humans and animals under different conditions such as inflammation, stress, medication, and nutrition. LPLs are particularly interesting because they are known to possess pro- and anti-inflammatory properties and may be generated by two different pathways: either by the influence of phospholipase A_2 or by different reactive oxygen species that are generated in significant amounts under inflammatory conditions. Both lead to the cleavage of unsaturated acyl residues. This review provides a short summary of the mechanisms by which lipid biomarkers are generated under *in vitro* and *in vivo* conditions. The focus will be on lysophosphatidylcholine (LPC) because usually, this is the LPL species which occurs in the highest concentration and is, thus, easily detectable by chromatographic and spectroscopic methods. Finally, the effects of lipid biomarkers as signaling molecules and their roles in different human and animal pathologies such as infertility, cancer, atherosclerosis, and aging will be shortly discussed.

Keywords: disease markers, inflammation, lysophospholipids, lysophosphatidylcholine, phospholipids, phospholipase, reactive oxygen species, disease marker

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INTRODUCTION – PHOSPHOLIPIDS AS EDUCTS OF RELEVANT PHYSIOLOGICAL MOLECULES

Phospholipids (PL) constitute an important class of biomolecules, among which glycerophospholipids (GPL) are of high relevance (Law et al., 2019). All GPLs consist of a glycerol backbone, where two hydroxyl groups are esterified with two (often varying) fatty acids. The third hydroxyl group is esterified with phosphoric acid. The resulting molecule is termed "phosphatidic acid" (PA). *Via* ester condensation with different alcohols such as choline or ethanolamine, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) are generated. These compounds represent the most abundant zwitterionic GPL in mammalian membranes.

The majority of PLs occurring under *in vivo* conditions are characterized by a saturated fatty acyl residue in *sn*-1 position, while the second fatty acyl residue is often mono- (e.g., oleic acid), di- (e.g., linoleic acid) or even higher unsaturated (e.g., arachidonic or docosahexaenoic acid, which contain four or six double bonds, respectively). GPLs are converted into lysophospholipids (LPLs) by the action of phospholipases. The reactions catalyzed by these enzymes are illustrated in **Figure 1**. The released free fatty acids have considerable physiological significance: highly unsaturated fatty acids such as arachidonic acid (C20:4) are very sensitive to oxidation and its metabolic (oxidation) products such as thromboxanes, prostaglandins, or leukotrienes have a considerable biological impact (Johnson et al., 2020).

Both, the LPL as well as the free fatty acid are considered as important molecules with potential messenger functions and destabilize cellular membranes due to their detergent-like character (Hu et al., 2007).

GENERATION OF LYSOPHOSPHOLIPID UNDER THE INFLUENCE OF PHOSPHOLIPASES AND REACTIVE OXYGEN SPECIES

Phospholipase "A" (PLA) is represented by a group of enzymes that catalyze the hydrolysis of one fatty acyl residue from the glycerol backbone of a PL. By this reaction, a free fatty acid is released and the corresponding LPL is left in the membrane (Peng et al., 2021; **Figure 1**).

The cleavage of phosphatidylcholine (PC) by PLA_2 yields lysophosphatidylcholine (LPC) and free fatty acids, e.g., arachidonic acid, the educt of eicosanoids. The hydrolysis of diacylglycerol (DAG) by DAG lipase at the *sn*-1 position results in 2-arachidonoylglycerol (2-AG) followed by a monoacylglycerol lipase (MAGL)-dependent hydrolysis of 2-AG to generate arachidonic acid and further eicosanoid pathway metabolites. Please note that more degradation products such as lysophosphatidic acid (LPA) (Hosogaya et al., 2008) (LPA) and glycerophosphocholine (GPC) can be produced by other phospholipases as well as reconversion by acyltransferases can take place (Brindley and Bräuer, 2010).

Based on the stereospecificity of the reaction, PLA_1 and PLA_2 activities can be differentiated. PLA_1 enzymes generally play a minor role than PLA_2 (Richmond and Smith, 2011), although there is increasing evidence that PLA_1 activity is underestimated regarding the generation of lysophosphatidylserine (LPS) (Iwata et al., 2021). The prevailing opinion is that LPLs are generated under *in vivo* conditions by the release and/or activation of PLA_2 that is particularly present in neutrophilic granulocytes, important cellular mediators of inflammation. However, neutrophils do not only secrete PLA_2, but are also capable of generating reactive oxygen species (ROS; Pérez-Figueroa et al., 2021). All ROS are derived from atmospheric air oxygen, which is converted in a set of reactions into H_2O_2. This is the precursor for the generation of further, much more reactive species, for instance, hydroxyl radicals ($HO^{\bullet}$). Despite the high reactivity of $HO^{\bullet}$, that reacts diffusion-controlled with nearly all organic molecules, another ROS seems to be responsible for the increased levels of LPC under pathological conditions: hypochlorous acid (HOCl; Schröter and Schiller, 2016). HOCl is generated under *in vivo* conditions from H_2O_2 and Cl^- ions under catalysis of myeloperoxidase (MPO; Kargapolova et al., 2021):

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^-$$

Myeloperoxidase is nearly exclusively found in neutrophils, where it amounts to approximately 5% of the total protein content (Schröter and Schiller, 2016). As the number of neutrophilic granulocytes increases massively under inflammatory conditions, the roles of MPO and its products are obvious (Arnhold, 2020). *In vitro*, it could be shown that LPC is also generated from isolated PC by the reaction with HOCl (Arnhold et al., 2002).

METHODS OF LYSOPHOSPHOLIPID DETERMINATIONS

Methods of LPL determinations often rely on, for instance, UV-, fluorescence, or ESR Spectroscopy, radioactivity or capillary electrophoresis. These methods detect mainly the related phospholipase activities, need prior labeling and/or do not give detailed structural information of the generated LPL. We will focus here on NMR (particularly ^{31}P and ^{1}H NMR), chromatography (mainly HPLC and TLC) and mass spectrometry as these methods overcome many disadvantages of the methods mentioned above – in particular, they do not require any specific labels. The advantages and drawbacks as well as details of the individual techniques are compared in **Table 1**.

It is important to note, that LPC can be generated from PC even under *in vitro* conditions. That is, even solutions of PC that are meant to be pure still often contain small amounts of LPC and the LPC moiety increases during storage (Hernandez-Caselles et al., 1990). Of course, the amount of detected LPC is also influenced by the applied extraction protocol because LPC is much more polar than other lipids. MALDI MS has the considerable advantage that LPC may also be determined from native samples, i.e., without the necessity of sample extraction that may result in the LPL loss. These aspects were recently discussed by Ditz and Fuchs (2018) and have already been comprehensively reviewed by Petković et al. (2005).

LYSOPHOSPHOLIPID AS INFLAMMATION AND DISEASE MARKERS

Lysophospholipid (LPL) such as LPC, LPA, sphingosine-1-phosphate (S1P), LPS (Kurano et al., 2015), and lysophosphatidylinositol (LPI) have pronounced effects on

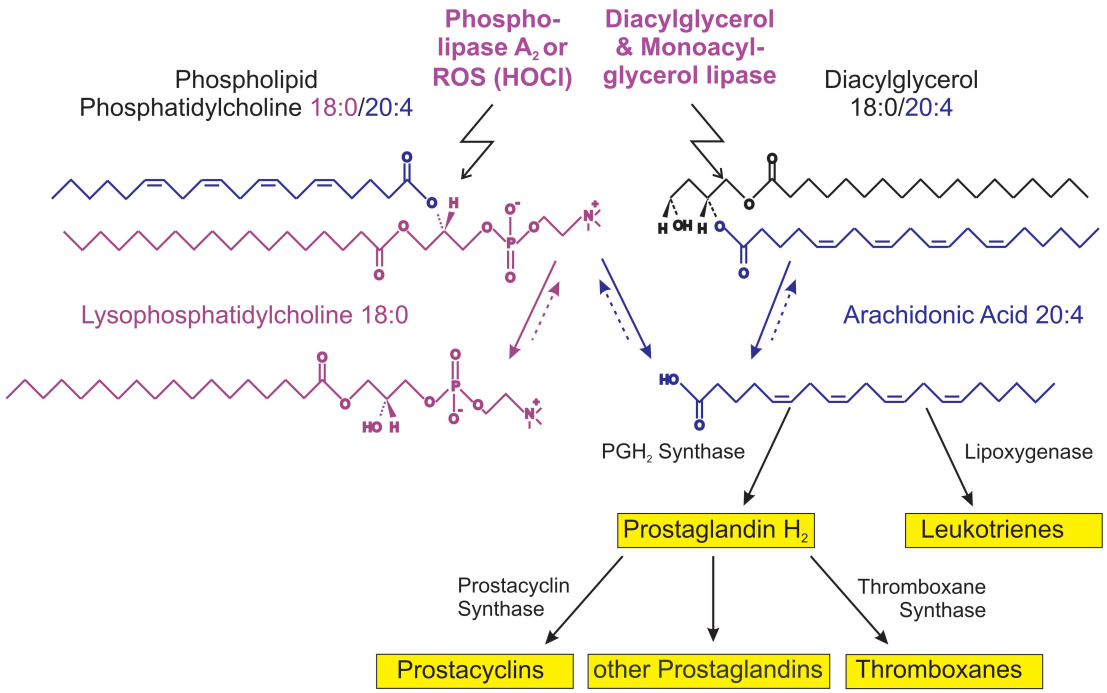

FIGURE 1 | Survey of lysophosphatidylcholine (LPC) metabolism.

diverse cell lines and the immunological effects induced by these compounds have already been reviewed (Xu et al., 2013). For some LPL, such as LPI and LPS signaling *via* G protein-coupled receptors (GPCR) has been described and was comprehensively reviewed by Tan et al. (2020). Even though the possibility of GPCR-mediated function of LPC has been pointed out, there is currently no GPCR for LPC binding known (Makide et al., 2014). Despite many studies revealed pro-inflammatory effects of LPL – such as LPC as key marker that is positively associated with cardiovascular and neurodegenerative diseases – there are also research articles demonstrating anti-inflammatory effects of these compounds, making findings controversial (Knuplez and Marsche, 2020). This controversial behavior can be explained by the *in vivo* generation of two different compounds: LPL and free fatty acids. In inflammation, the released free fatty acid is often arachidonic acid that is readily converted into compounds with immunomodulatory effects. Furthermore, LPC is a precursor of extracellular LPA, an important lipid mediator and modulator of neuronal function (Brindley and Bräuer, 2010). LPA is produced from LPC by autotaxin and is therefore a direct executioner of LPC (Hosogaya et al., 2008; Yatomi et al., 2018). However, the concentration of LPA in body fluids and tissues is always much lower (normally about two orders of magnitude) compared to LPC. In a nutshell, both, the generation of LPL and the formation of arachidonate-derived metabolites must be inhibited for pharmacological effects (Sun et al., 2014).

There is one interesting study that illustrates the potential "marker quality" of LPC: it was shown that horse sera with a high LPC content are not useful for cell experiments because they have deleterious effects on cell growth. This suits the finding that horse sera with moderate amounts of LPC were also characterized by low levels of inflammatory eicosanoids (Ditz et al., 2019).

Lysophosphatidylcholine Under Inflammation and Stress

The concentration of LPC increases under inflammatory conditions. This has been shown, for instance, in joint fluids from rheumatoid arthritis patients (Fuchs et al., 2005) or atherosclerosis patients (Matsumoto et al., 2007). Under certain conditions, however, the opposite was observed: plasma LPC showed an inverse relationship with cardiovascular diseases (Ganna et al., 2014). In other studies, the LPC/PC ratio in plasma as well as cerebrospinal fluid from patients with Alzheimer's disease decreased (Lin et al., 2017). Unfortunately, all these studies were methodologically different and, thus, the comparability is poor.

Lysophosphatidylcholine and LPE levels rise considerably in human inflammatory liver tissue (Schober et al., 2009). Particularly, LPC could serve as a biomarker in fatty liver disease. However, it is known that also a metabolically healthy fatty liver exists (Stefan et al., 2019). The comparison of molecule pattern of plasma from insulin-sensitive and insulin-resistant human subjects have shown that particularly LPCs are capable to distinguish benign and malignant non-alcoholic fatty liver (Lehmann et al., 2013) and mechanisms involved in this process, e.g., pro-inflammatory signaling (*via* regulation of LPC acyltransferase) were discussed. Similarly, in studies with non-alcoholic steatohepatitis in mice major reductions in LPC 16:0, 18:0 and 18:1 were identified (Tanaka et al., 2012).

TABLE 1 | Survey of selected techniques of lysophospholipid (LPL) analysis.

	Principle	Advantages	Drawbacks	Remarks
High-performance liquid chromatography (HPLC)	Separation on a "stationary phase" under high pressure by elution with solvents of different polarities; "reversed" phase is more common than normal phase.	Can be standardized; protocols are available for many analytes; coupling with MS is well established - although detection by UV or light scattering is still widely used; preparative HPLC is possible.	Requires experienced operators; detection of saturated lipids (lack of UV absorptions) is difficult; post-column derivatization is time-consuming.	Routine method in many laboratories; "fine-tuning" of the mobile phase to the relevant sample is normally required; a timely review with the focus on liposomes and the detection of LPC as impurity is available in de Magalhães Benedetti et al. (2020).
Thin-layer chromatography (TLC)	Separation on a stationary phase (normally silica gel) due to polarity differences of the analytes; different modifications/polarities of the stationary phase are commercially available	Inexpensive and fast; many samples can be simultaneously analyzed; stainings with different properties (non-covalent, covalent binding, UV, fluorenscence detection) can be used for detection.	Oxidation (of unsaturated LPL species) may occur during the run; resolving LPL with different acyl residues is difficult; less sensitive compared to MS.	Often used as initial method if a complex lipid mixture has to be analyzed in detail; TLC-based lipid analysis is still common (Adibi et al., 2018).
ESI MS	Ions are generated from charged droplets.	"very soft" ionization method; little analyte fragmentation; quantification possible in the presence of a suitable internal standard.	Ion suppression may occur; strongly affected by sample impurities as well as the composition of the solvent.	ESI MS is already in clinical use since metabolite determination by MS is often cheaper than other methods (Liebisch et al., 2002).
MALDI MS	Ions are generated by laser irradiation of a matrix/analyte cocrystal	"soft" ionization method; little analyte fragmentation; very little sample pretreatment and purification required.	Ion suppression may occur; obtaining quantitative data is difficult.	PC/LPC ratios are often given which can be calculated without internal standards (Bresler et al., 2011).
^{1}H NMR	Differences in electron densities lead to different chemical shifts of the observed nucleus within a given compound	Basically all lipids are detectable; isomers can be differentiated without the need of previous separation; spectra exhibit quantitative information.	Analyses of mixtures lead to complex spectra; need of deuterated solvents; expensive equipment; differences in the fatty acyl compositions can hardly be resolved.	One characteristic functional group (the quaternary ammonia group) is used as the sensor to detect the different classes; high magnetic field strengths are required to resolve PC, LPC and SM (same headgroups) (Soininen et al., 2007).
^{31}P NMR	The different chemical environment renders each P atom a characteristic chemical shift.	Direct absolute quantitation is possible; isomeric lipids can be differentiated.	Limited sensitivity (order of magnitude less than MS); requires high amounts of sample; expensive equipment.	Detergents or solvent mixtures have to be used in order to suppress the aggregation of phospholipids; acyl migration may falsify the results (Sugasini and Subbaiah, 2017); has been recently reviewed: (Li et al., 2017).

The advantages and drawbacks of the different methods are summarized. Note that chromatographic separation is often combined with MS detection. A review dealing with selected methods is available in Helmerich and Koehler (2003).

It could also be shown that LPC enhances the generation of superoxide anion radicals ($O_2^{\bullet -}$) as well as H_2O_2 (Englberger et al., 1987), i.e., LPC may trigger the generation of even stronger ROS such as $HO^{\bullet}$ or HOCl. Similar observations were made for lymphocytes, at which the presence of LPC led to an increased number of apoptotic cells. However, it is not known whether these studies are relevant under *in vivo* conditions. *In vivo*, there is a huge amount of proteins with high affinity for LPC, e.g., albumin and lipoproteins. Thus, the amount of physiologically available LPC may vary considerably. Anyway, LPC normally does not accumulate in the body because different mechanisms limit the elevation of LPC concentrations: (I) the re-acylation of LPC to PC, (II) the degradation of LPC to GPC by the cleavage of the fatty acyl residue in the *sn*-1 position by lysophospholipases. GPC lacks both acyl residues and is, thus, soluble in water. An overview of the PC- and LPC-related pathways was provided by Fuchs et al. (2012).

Despite the many open questions, targeting LPC and its metabolic pathways might be a prospective treatment strategy of inflammatory diseases (Liu et al., 2020).

Lysophospholipid in Fertility and Infertility

Male gametes are perhaps the most important cells at which LPL are known to have significant impact. The related process is called "capacitation": mammalian spermatozoa undergo a variety of physiological events, which make them ready for fertilization, i.e., the fusion between the sperm and the female oocyte.

Ye (2008) reviewed the physiological functions of LPL (particularly LPA and S1P) in reproduction as well as potential pathological side effects. LPC has been shown to have an impact on human corporal smooth muscle cells, and therefore, might lead to an impaired penile function through TPR channels (So et al., 2005). Furthermore, it is known since many years that LPC is capable of triggering sperm acrosomal exocytosis, an important event that primes spermatozoa for successful fertilization (Ehrenwald et al., 1988). Roldan and Shi (2007) examined the action of PLA_2 and its role for successful fertilization. There are two obvious functions of the generated LPL: on the one hand, they represent second messengers for cellular signaling. On the other hand, they act as detergents and destabilize the membranes by changing their biophysical properties. Despite the obvious physiological role of PLA_2 activation, spermatozoa avoid premature destabilization and, thus, a carefully controlled equilibrium between LPL generation and its reacylation into the corresponding PL must exist. Deviations from normal conditions may otherwise lead to pathological situations, so that an enhanced sperm LPL concentration is presumably indicative of a reduced fertilizing potential. There is evidence that the LPC content reciprocally correlates with the sperm quality of human sperm (Roldan and Shi, 2007). Using magnetic assisted cell sorting (MACS) to separate intact and apoptotic (annexin V positive) spermatozoa, it was shown that the LPC content is much higher in the apoptotic sperm (Glander et al., 2002). Although it is not yet clear whether the observed effect is triggered by an enhanced generation of ROS or an elevated PLA_2 activity, there are many indications that oxidative stress has a pronounced impact on sperm physiology (Takeshima et al., 2020). For instance, sperm from extremely obese men are characterized by an enhanced LPC content. This correlates with a reduced fertilizing capacity of these men (Pyttel et al., 2012).

Thus, LPC might represent an interesting "marker" molecule in fertility that definitely is worth of further investigations because it has a considerable advantage: in comparison to proteins, LPC is a non-specific marker that could be useful for human as well as animal spermatozoa (Nimptsch et al., 2014).

Lysophosphatidylcholine and Aging

It is known that lipids play a role in lifespan regulation and age-related diseases (de Diego et al., 2019). Very recently, the use of lipidomics to age-related musculoskeletal disorders was reviewed (Mo et al., 2021). Siddharth et al. (2017) monitored changes of serum lipids in aged mice with a sarcopenic phenotype and found that levels of LPC 20:5 and LPC 20:3 were reduced – although an explanation of the occurrence of these unusual fatty acids was not provided. In humans blood LPC levels tend to decrease with age: low plasma levels of LPC are associated with impaired mitochondrial oxidative capacity in adults (Semba et al., 2019). Lower levels of LPC 18:2 were shown to be predictive of memory impairment and enable the prediction of a greater decline of gait speed in the elderly (Gonzalez-Freire et al., 2019).

Patients with cancer, an age-related disease, exhibit a decreased LPC concentration in plasma (Taylor et al., 2007). Similarly, Kim et al. (2014) found that the LPC 24:0 levels in aged mice were lower compared to young mice. Although no explanation of the occurrence of this less common organic residue (lignoceric acid) is provided, the authors conclude that the ratios of the individual metabolites PC and LPC could serve as potential biomarkers for aging and age-related diseases. In contrast, there are also studies showing that during aging the levels of LPC increase – in particular in patients suffering from Alzheimer's disease (Dorninger et al., 2018). Although not yet carefully studied, LPC seems to enhance oxidative stress *via* the 5-lipoxygenase pathway in rat aorta during aging (Zou et al., 2009). This might foster further pharmacological studies because lipoxygenase is one interesting target for many drugs.

Lysophosphatidylcholine Under Medication, Nutrition, and Pharmacological Aspects

The normal concentration of LPC in plasma is significant (about 200–300 μM) – with most LPC bound to albumin and lipoproteins (Kishimoto et al., 2002). Anyway, LPC-treatment of mice induces enhanced phagocytic activity of macrophages (Lee et al., 2018). Intracutaneous injection of LPC in healthy volunteers similarly elicited acute inflammation with the accumulation of T lymphocytes, monocytes, and neutrophils (Murugesan et al., 2003). Direct pro-inflammatory and atherogenic effects of LPC have become apparent over the past 30 years. Nowadays, there is increasing evidence that LPC has also anti-inflammatory properties, making its profile more complex than initially assumed (Schilke et al., 2020). These controversial effects of LPC are presumably caused by differences in length and/or the saturation state of the fatty acyl chain (Tan et al., 2020). Additionally, different functional effects of LPC may also be due to different moieties of the free and the albumin-bound LPC (Mehta, 2005).

Dietary polar lipids are relevant for the cognitive development and are distributed throughout the body by lipoproteins (Zheng et al., 2019). Lipids play an unequivocal active part in the acceptability, flavor, and perception of foods and may be beneficial for health – or lead to various pathologies (Meynier and Genot, 2017). Finally, LPC is used in animal nutrition to improve animal performance, i.e., to favor the digestion of food and the release of nutrients from the diet (Wealleans et al., 2020).

As LPC concentration is elevated in many pathologies, different attempts were undertaken to decrease the LPC concentration *in vivo*. Because of the obvious contribution of PLA_2, this has raised interest for pharmacologically-active substances capable of inhibiting PLA_2 activity (Yalagala et al., 2019; Frangieh et al., 2021). However, PLA_2 activation does not only result in LPC generation but also in arachidonate-derived free radical intermediates (Sun et al., 2014) and further ROS. Therefore, a single drug molecule with both – anti-oxidative and PLA_2 inhibitory activity – would be useful since it could inhibit PLA_2 activity and simultaneously scavenge free radicals and lipid peroxides which are released during C20:4 metabolism.

CONCLUDING REMARKS

Lysophospholipids represent lipid molecules with a Janus-faced character: on the one hand, LPL are important signaling molecules that are required for physiological processes, such as successful fertilization or proper memory function. On the other hand, their *in vivo* concentrations has to be carefully controlled, e.g., a disturbance of the equilibrium between LPC generation and re-acylation can lead to severe pathological conditions. The focus of this review is on LPC and this is also true for previous studies. This is, at least partially, caused by the fact that this LPL can be most sensitively detected. This can be achieved with low resolution mass spectrometers. Furthermore, LPC is a comparably stable compound, that does not react with other compounds that is again an excellent property for its successful determination. It remains to be elucidated whether and which LPL are useful and reliable biomarkers of (inflammatory) diseases. Contradicting effects of LPC observed in experimental models and patient samples could be due to differences in saturation and/or length of the fatty acyl chain.

AUTHOR CONTRIBUTIONS

JS wrote chapter 3 including **Table 1**. KE wrote chapter 4.2. BF wrote chapter 2 including **Figure 1** and chapter 4.3. CG wrote chapter 4.4. All authors contributed to the other chapters, abstract, introduction, and conclusions.

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A Lipidomic Approach to Identify Potential Biomarkers in Exosomes From Melanoma Cells With Different Metastatic Potential

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Melanoma, one of the most lethal cutaneous cancers, is characterized by its ability to metastasize to other distant sites, such as the bone. Melanoma cells revealed a variable *in vitro* propensity to be attracted toward bone fragments, and melanoma-derived exosomes play a role in regulating the osteotropism of these cells. We have here investigated the lipid profiles of melanoma cell lines (LCP and SK-Mel28) characterized by different metastatic propensities to colonize the bone. We have purified exosomes from cell supernatants by ultracentrifugation, and their lipid composition has been compared to identify potential lipid biomarkers for different migration and invasiveness of melanoma cells. Matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) lipid analysis has been performed on very small amounts of intact parental cells and exosomes by skipping lipid extraction and separation steps. Statistical analysis has been applied to MALDI mass spectra in order to discover significant differences in lipid profiles. Our results clearly show more saturated and shorter fatty acid tails in poorly metastatic (LCP) cells compared with highly metastatic (SK-Mel28) cells, particularly for some species of phosphatidylinositol. Sphingomyelin, lysophosphatidylcholine, and phosphatidic acid were enriched in exosome membranes compared to parental cells. In addition, we have clearly detected a peculiar phospholipid bis(monoacylglycero)phosphate as a specific lipid marker of exosomes. MALDI-TOF/MS lipid profiles of exosomes derived from the poorly and highly metastatic cells were not significantly different.

Keywords: melanoma, membrane vesicles, osteotropism, lipids, MALDI-TOF/MS

Abbreviations: 9-AA, 9-Aminoacridine hemihydrate; BMP, bis(monoacylglycero)phosphate; CL, cardiolipin; CHO, cholesterol; EMT, mesenchymal transition; EV, extracellular vesicles; GSLs, glycosphingolipids; GPs, glycerophospholipids; GSLs, glycosphingolipids; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; Pls, plasmalogens; PS, phosphatidylserine; PSD, post source decay; R_f, retention factors; SM, sphingomyelin; SPs, sphingophospholipids; TEM, transmission electron microscopy; TLC, thin-layer chromatography.

INTRODUCTION

Living cells release lipid bilayer vesicles into the extracellular fluid, i.e., extracellular vesicles (EVs), which aroused great interest in the scientific community in the last 10 years due to their important role in complex intercellular communication.

Extracellular vesicles are released by cells of most living organisms (animals, bacteria, and plants) and are different in origin, size, and composition. They are grouped in microvesicles, apoptotic bodies, and exosomes. Microvesicles ranged between 150 and 1,000 nm in diameter and are formed by outward budding of the plasma membrane, while apoptotic bodies (over 1,000 nm) are released when plasma membrane blebbing occurs during apoptosis. Exosomes refer to smaller vesicles ranging from 30 to 150 nm and differ because of their endocytic origin. Endocytosis from the plasma membrane leads to the formation of an early endosome in the cytosol, and then the inward budding of the endosomal membrane fills its lumen with intraluminal vesicles, which mature in late endosome, termed also as multivesicular body (MVB). Their fusion with the plasma membrane allows for the release of intraluminal vesicles into the extracellular space while their different origin determines variation in lipid bilayer membrane. Their common feature, however, is that they carry a pool of functional biomolecules (including DNA, RNAs, proteins, and lipids) (Record et al., 2014; Isola et al., 2016; Kalluri, 2016; Kalra et al., 2016; Skotland et al., 2017; Kalluri and LeBleu, 2020).

Exosomes were described for the first time in 1983 in a study about the externalization of the transferrin receptor during maturation of sheep reticulocytes (Pan and Johnstone, 1983). It has been well-established that they are only produced from viable cells and play an important role in cell-to-cell communication through the transfer of their cargo.

Although both normal and pathological cells are able to secrete exosomes, cancer cells generally secrete more exosomes than normal counterparts (Tickner et al., 2014). Exosomes derived from cancer cells modify local and distant microenvironments and promote metastasis differently, as influencing the immune system, promoting epithelial to mesenchymal transition (EMT), angiogenesis, and organotropism (Zhang and Yu, 2019). The evidence of an exosomal role in cancer is widely reported, but the complex process by which exosomes promote oncogenic progression and metastases is yet to be clarified. Cancer-derived exosomes may establish a favorable milieu for cancer progression by carrying oncogenic cargo of proteins (Rajagopal and Harikumar, 2018). Some comparative studies on protein content between metastatic and non-metastatic exosomes have been reported thus revealing different protein expressions (Peinado et al., 2012; Garnier et al., 2013; Jeppesen et al., 2014).

It is noteworthy that high synthesis activity of the lipids is required for the proliferation of tumor cells to generate new biological membranes. Although the physiological mechanisms are not yet understood, the lipid synthesis in malignant tissues plays a critical role during tumorigenesis as the cell transformation, development, and tumor progression (Baenke et al., 2013).

On the other hand, the lipid characterization of different tumor cell lines has demonstrated the enrichment of some lipid species in contrast to the decrease of others, as compared to normal cells (Hilvo et al., 2011; Lee et al., 2012; Bandu et al., 2018; Messias et al., 2018; Szlasa et al., 2020). In this context, an important role is attributed to the higher level of saturated and monounsaturated phospholipids in cancer cells that can prevent lipid peroxidation and thus protect them from oxidative damage (Rysman et al., 2010).

It remains to be clarified how changes in lipid composition can affect the metastatic potential of cancer cells. In order to shed light on this, a detailed analysis of tumor tissue is required to determine the full spectrum of lipids within tumor cells. To this end, lipidomic approaches are often applied to analyze the lipid composition of tumor cell lines or fresh tumor samples by mass spectrometry (Hilvo et al., 2011; Bandu et al., 2018). Nevertheless, lipidomic characterization of tumor-derived exosomes, including melanoma, is still poorly investigated.

Melanoma cells, characterized by their ability to metastasize to distant sites, create a favorable environment for their growth by activating the EMT and favoring the immune system evasion (Sceneay et al., 2013; Tucci et al., 2014; Passarelli et al., 2017).

In a recent study, Mannavola et al., have investigated the potential role of melanoma-derived exosomes in favoring the motility and invasiveness of cells toward bone fragments by activating the SDF-1/CXCR4/CXR7 chemotactic axis under the influence of SDF-1 gradient. The authors found that poorly (LCP) and highly (SK-Mel 28) metastatic melanoma cells had variable propensity to be attracted toward the bone *in vitro*. As the migratory capacity of LCP cells increased once exposed to the stimuli of bone fragments, they are defined as "osteotropic," by contrast, as bone fragments exerted only a modest effect on SK-Mel28 cells, they are named "not-osteotropic." Hence, tumor-derived exosomes reprogram the "innate" osteotropism of melanoma cells by upregulating the membrane receptor CXCR7 (Mannavola et al., 2019).

This study aims at exploring the lipid content of both LCP and SK-Mel28 melanoma cells and exosomes in order to identify potential lipid biomarkers for their different migration behavior and invasiveness. We compare the lipid content of whole melanoma cells and exosomes by direct matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) lipid analysis of intact membranes. This approach is crucial for investigating by statistics analysis exosome membrane lipids. In parallel, the classical semiquantitative analysis of extracted lipids by TLC coupled to MS has been completed with the purpose to improve knowledge derived from a direct method and, especially, to clearly identify the peculiar phospholipid pattern in melanoma-derived exosomes.

MATERIALS AND METHODS

Materials

All organic solvents used for lipid extraction and MS analyses were commercially distilled and of the highest available purity (Sigma–Aldrich). The matrix used for MALDI-TOF/MS

analyses was the 9-Aminoacridine hemihydrate (9-AA) and was purchased from Acros Organics (Morris Plains, NJ, United States). The following commercial glycerophospholipids (used as standards): 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol), bis(monooleoylglycero)phosphate, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate, bis(monomyristoylglycero) phosphate, N-palmitoyl-D-erythro-sphingosylphosphorylcholine, N-stearoyl-D-erythro-sphingosylphosphorylcholine, *sn*-(3-myristoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3′-(1′,2′-dimyristoyl)-glycerol, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol), 1,2-dimyristoyl-*sn*-glycero-3-phosphate, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine, 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol, 1′,3′-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol, were purchased from Avanti Polar Lipids, Inch (Alabaster, AL, United States). Plates for thin-layer chromatography (TLC) (Silica gel 60A, 10 × 20 cm, 0.2-mm-thick layer), were purchased from Merck (Darmstadt, Germany).

Exosomes Isolation From Melanoma Cells and Characterization

Melanoma (SK-Mel28 and LCP) cell lines (ATCC, Rockville, MD, United States) were cultured in an exosome-free complete medium as previously described (Mannavola et al., 2019).

Exosomes were purified by ultracentrifugation of supernatants from 48-h cultured melanoma cells at 80% confluency (Théry et al., 2006). Extracellular vesicles were isolated from a 100-ml culture medium using, initially, three different sedimentation speeds: 300 × *g* for 10 min to remove cells, 2,000 × *g* to remove dead cells and debrides, and 10,000 × *g* to remove microvesicles. Next, the supernatant was centrifuged at 100,000 × *g* for 70 min two times at 4°C to collect exosomes. They were then resuspended in PBS and stored at −80°C.

After isolation, the characterization of exosomes has been performed exactly following the methods as previously described in Mannavola et al. (2019). Briefly, exosome preparations were verified by measuring the expression of CD63, CD81 (eBioscience), and CD9 (BD Pharmingen) by flow-cytometry (Tucci et al., 2018; Pezzicoli et al., 2020). Moreover, to further validate the purity of exosome preparations, western blots were performed to measure the levels of CD81, TSG101, calnexin, and bovine serum albumin in accordance with Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines (Théry et al., 2018). The transmission electron microscopy (TEM) defined the morphology of vesicles.

Lipid Extraction

Total lipids of LCP and SK-Mel28 cells and exosomes were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959); the extracts were carefully dried under N_2 before weighing and then dissolved in chloroform (10 mg/ml). Usually, about 1 mg of total lipids was obtained from about 13 million cells of each cell line. In the present study, we have analyzed lipids extracted from three different preparations for each tumor cell line.

Thin-Layer Chromatography Analyses (TLC)

Total lipid extracts were analyzed by TLC on silica gel 60A plates (Merck, 20 × 10 cm, layer thickness, 0.2 mm). The plates were washed two times with chloroform/methanol (1:1, by vol.) and activated at 180°C before use. Polar lipids were eluted with an acid solvent (chloroform/methanol/acetic acid/water, 85:15:10:3.5, by vol.). Total lipid detection was carried out by molybdenum blue spray reagent (Sigma-Aldrich) specific for phospholipids (Kates, 1986). Alternatively, total lipid detection was done with reversible staining exposing the TLC plate to iodine vapor for 4–5 min for staining all classes of lipids before the lipid bands isolation. To analyze in detail the various components of the lipid extracts, each band present on the plates was scraped and lipids extracted from silica, as previously described (Kates, 1986); briefly, 0.5 ml of a mixture chloroform/methanol/water (1:2:0.8, by vol.) has been added to silica bands, and the samples were vigorously stirred and centrifuged. Lipid bands of preparative TLC were analyzed by positive and negative ion mode MALDI-TOF/MS.

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight/Mass Spectrometry (MALDI-TOF/MS)

MALDI-TOF mass spectra were generally acquired in the negative and positive ion modes on a Bruker Microflex LRF mass spectrometer (Bruker Daltonics, Bremen, Germany). The system utilized a pulsed nitrogen laser, emitting at 337 nm, the extraction voltage was 20 kV, and gated matrix suppression was applied to prevent detector saturation. For each mass spectrum, 2,000 single laser shots (sum of 4 × 500) were averaged. The laser fluence was kept about 5% above threshold to have a good signal-to-noise ratio. All spectra were acquired in a reflector mode (detection range: 200–2,000 mass/charge, *m/z*) using the delayed pulsed extraction; spectra were acquired in negative and positive ion modes. Peaks areas, spectral mass resolutions, and signal-to-noise ratios were determined by the software for the instrument *Flex Analysis 3.3* (Bruker Daltonics).

A mix containing 1,2-dimyristoyl-sn-glycero-3-phosphate, 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol), sn-(3-myristoyl-2-hydroxy)-glycerol-1-phospho-sn-3′-(1′,2′-dimyristoyl)-glycerol, 1′,3′-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol, GM1 Ganglioside was always spotted next to the sample as external standard, and an external calibration was performed before each measurement in a negative ion mode; the mass range of the authentic standards is 590–1,550 atomic mass units (amu). A mix containing 1,2-distearoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine, 1,2-di-O-phytanyl-sn-glycero-3-phosphocholine was always spotted next to the sample as external standard, and an external calibration was performed before each measurement in a positive ion mode; the mass range of the authentic standards is 670–1,350 atomic mass units (amu).

For the analysis of lipid extract, the samples for MALDI-TOF analysis were prepared as previously described (Sun et al., 2008). Briefly, the total lipid extracts (10 mg/ml) were diluted from 20

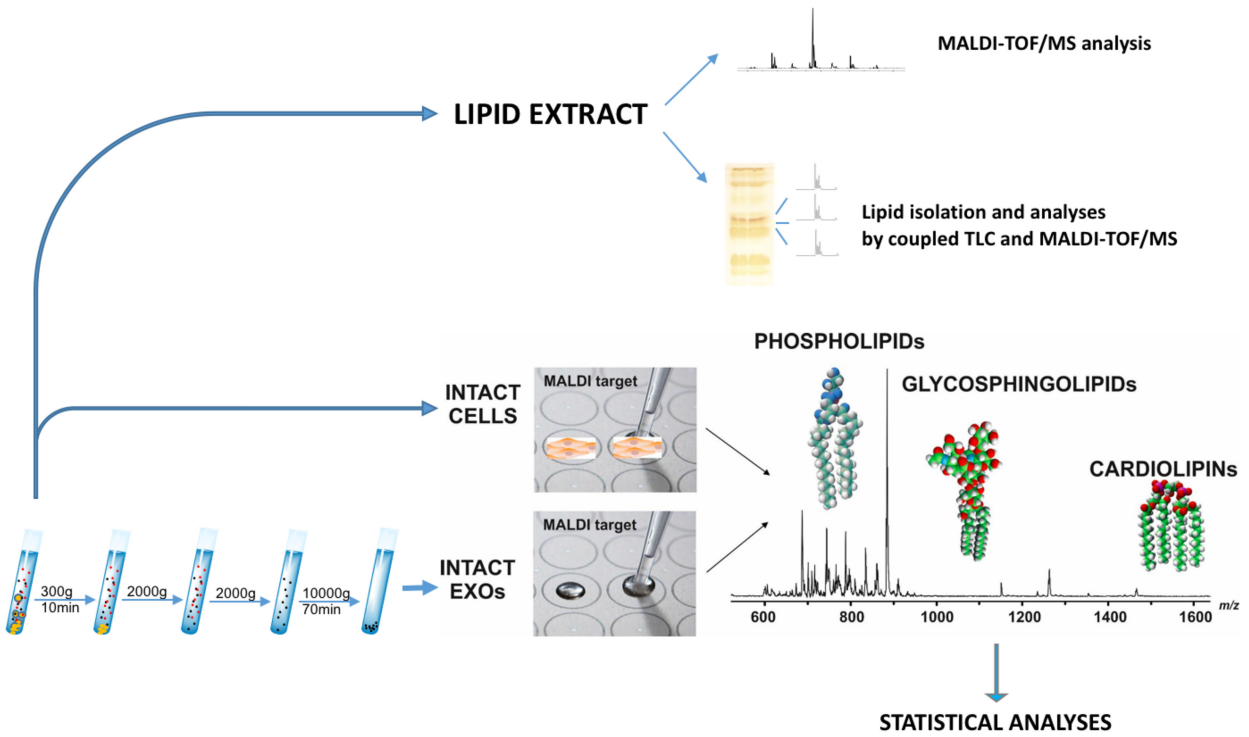

FIGURE 1 | Lipidomics workflow for melanoma cells and exosomes. Lipid extract from melanoma cells has been analyzed by MALDI-TOF/MS and coupled TLC and MALDI-TOF/MS. After exosomes have been purified from supernatants of LCP and SK-Mel28 cells, small amounts of intact exosomes and cells were loaded on the MALDI target and lipids were directly analyzed by MS in order to perform statistical analysis.

to 200 μl with a 60:40 (by vol.) 2-propanol/acetonitrile mixture. Next, 10 μl of a diluted sample was mixed with 10 μl of 9-AA (10 mg/ml). Then, 0.35 μl of the mixture was spotted on the MALDI target.

Lipids from intact melanoma cells and exosomes produced were directly analyzed by the "intact method," as previously described (Angelini et al., 2012). In brief, the cellular suspensions were syringed and centrifuged at 100,000 × *g* for 1 h and 10 min; the samples were all diluted to 0.5 μg/μl of total cellular protein concentration, determined by the Bradford method. Afterward, 1 μl of cellular suspension was spotted on the MALDI target (Micro Scout Plate, MSP 96 ground steel target). After water evaporation, a thin layer (0.35 μl) of matrix solution (9-AA 20 mg/ml in 2-propanol/acetonitrile, 60/40, by vol.) is then spotted in the dried sample. Finally, even after evaporation of the matrix, it is possible to analyze the sample directly with MALDI-TOF/MS.

In our study, mass spectra of three independent biological samples (i.e., three cell cultures and the corresponding lipid extracts) were considered to confirm reproducibility of the results.

In particular, series of MALDI mass spectra (three replicates for each sample) have been averaged by using the software for the instrument *CliProTools* 3.0 (Bruker Daltonics) in order to find the area under the peaks. The samples were analyzed, comparing different series of spectra from the two cell lines and the exosomes preparations. A *p*-value from paired Student's *t*-test < 0.05 was set as the threshold to define significant differences between the series of spectra.

Post Source Decay (PSD) spectra were acquired on a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany), as previously described (Fuchs et al., 2007). Briefly, the precursor ions were isolated using a time ion selector. The fragment ions were refocused onto the detector by stepping the voltage applied to the reflectron in appropriate increments. This was done automatically by using the "FAST" (fragment analysis and structural TOF) subroutine of the Flex Analysis software. Mass accuracy of our instrument is 200 ppm (external calibration). A specific lipid database (Lipid Maps Database, LIPID MAPS Lipidomics Gateway) (LIPID MAPS) was used to facilitate and confirm the assignment of lipid species.

RESULTS

The overall workflow employed here for the comprehensive lipidome analysis of LCP and SK-Mel28 cell lines and exosomes is shown in **Figure 1**. As previously described, total lipids of LCP and SK-Mel28 cells were extracted by the classical extraction method and analyzed by both MALDI-TOF/MS and coupled TLC and MALDI-TOF/MS.

In general, although main lipids are detectable by MALDI-TOF/MS, some lipid classes are more sensitively detected than others; consequentially, the signals of less sensitively

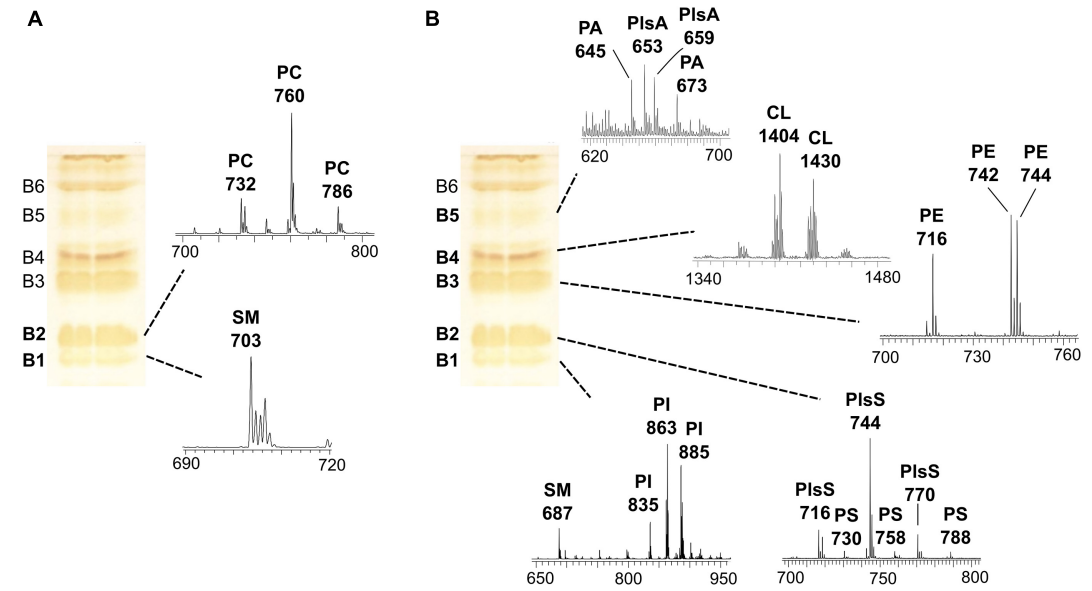

FIGURE 2 | MALDI-TOF/MS analysis of individual lipid bands isolated from melanoma cells by TLC. The total lipid extract of cells (LCP cell line) was loaded on the plate (120 μg per each lane); TLC was stained with iodine vapors (temporary staining of all classes of lipids). Six lipid bands (B1–B6) were marked with a pencil, and silica was scraped; lipids were extracted from silica and analyzed by MALDI-TOF/MS. The same TLC plate is shown on the left of both panels. **(A)** shows the MALDI-TOF/MS spectra of the lipid bands acquired in positive ion mode, while **(B)** shows the mass spectra of the lipid bands acquired in negative ion mode.

detected phospholipids are suppressed, when complex mixtures are analyzed. Therefore, a pre-chromatographic separation of different lipid classes by TLC, followed by MALDI-TOF/MS analysis of individual lipid bands, offers the opportunity to identify minor components as well, whose signals were barely distinguishable from the noise in the total lipid profiles.

Then, in order to perform statistical analysis, MALDI-TOF/MS was used to directly acquire lipid profiles of the two melanoma cell lines and their purified exosomes. This "intact method" is highly sensitive and crucial to analyze only minute amounts of biological material; moreover, it is possible to directly load 1 μl only of an intact sample (at 0.5 μg/μl protein concentration) on the MALDI target, having a fast and detailed lipidomic analysis, reducing the processing time of the samples (Angelini et al., 2012).

Analysis of Total Lipid Extracts of Melanoma Cell Lines by Coupled TLC and MALDI-TOF/MS

In order to detect main lipid species, those that ionize better in a negative ion mode (i.e., acidic lipid classes) and others that give intense signals in a positive ion mode (i.e., zwitterionic glycerophospholipids), depending on their chemical structures, we acquired the MALDI mass spectra of the total lipid extracts of melanoma cells in both negative and positive ion ionization modes. By comparing the MALDI lipid profiles of the two cell lines, we could not observe qualitative differences (**Supplementary Figure 1**).

In order to gain detailed information on lipid species, the coupled TLC and MALDI-TOF/MS analysis was performed. First, the total lipid extract was analyzed by TLC, and lipids were stained by iodine vapors in order to isolate and purify the individual components (see plates on the left of the **Figures 2A,B**). Then, the various lipid bands were isolated by preparative TLC and analyzed by MALDI-TOF/MS (mass spectra of lipid bands are on the right of the plates in **Figures 2A,B**). By TLC analyses, individual lipids were identified by comparison of their retention factor (R_f) values with those of standards and by their response to specific staining (not shown).

In particular, six bands (B1–B6) of lipids were scraped from the plate, and then lipids were extracted from silica (Bligh and Dyer, 1959) and analyzed by MALDI-TOF/MS in positive and negative ion modes.

Figure 2A shows the MALDI-TOF mass spectra of lipid bands B1 and B2 in a positive ion mode, while **Figure 2B** shows mass spectra of bands B1–B5 in a negative ion mode.

A detailed list of main peaks detected in the MALDI-TOF mass spectra acquired in negative and positive ion modes, corresponding to the lipid species present in the lipid bands and/or in the total lipid extracts, is reported in **Tables 1**, **2**, respectively.

The sphingomyelin (SM) was detected in the band of the lowest retention factor on TLC (B1): positive ion mode MALDI-TOF/MS analysis of B1 revealed a main peak at *m*/*z* 703.5, while negative ion mode analysis of the same band revealed a signal at *m*/*z* 687.6 (see B1 mass spectra in **Figures 2A,B**). Both the signals correspond to the same species of SM, having a palmitic acid.

B1, analyzed in a negative ion mode, shows peaks attributable to the phospholipid phosphatidylinositol (PI), where the main

peaks are at *m*/*z* 835.6, 863.6, and 885.5 referable to different PI species (**Figure 2B**).

The mass spectrum of B2, in a positive ion mode, shows main peaks at *m*/*z* 732.5, 760.5, and 786.5, corresponding to protonated form of phosphatidylcholine (PC) species with different chain lengths (**Figure 2A**). A MALDI negative ion mode of the same band B2 shows peaks attributable to phosphatidylserine (PS) and some plasmenylserine (PlsS) species; the peaks at *m*/*z* 716.5, 744.6, and 770.5 can be assigned to the PlsS species, while the peaks at *m*/*z* 730.5, 758.4, and 788.6 refer to PSs (**Figure 2B**).

B3, analyzed in a negative ion mode, shows peaks (at *m*/*z* 716.5, 742.5, and 744.6) attributable to phosphatidylethanolamine (PE) species (**Figure 2B**).

In the mass spectrum of B4, in a negative ion mode, the peaks in the high range *m*/*z* 1,300–1,500 were assigned to cardiolipin (CL) species, having four acyl chains (at *m*/*z* 1404 and 1430) (**Figure 2B**).

B5, analyzed in a negative ion mode, shows low signals (at *m*/*z* 645.4 and 673.4) attributable to phosphatidic acid (PA) species; the peaks at *m*/*z* 653.3 and 659.5 can be assigned to plasmalogens species (PlsA).

B6 was assigned to the neutral lipid cholesterol (CHO) by comparing its R_f value on TLC with that of lipid standard, although we could not confirm its identity by MALDI-TOF/MS.

The use of coupled TLC and MALDI-TOF/MS analysis gave also the opportunity to identify and validate the assignments of minor lipids present in melanoma cells, such as PS, CL, and plasmalogen species. In conclusion, the TLC lipid profile of melanoma cells consists (in R_f order) of SM/PI, PS/PC, PE, CL, PA, and CHO.

Comparative Lipid Analysis of Melanoma Cells and Exosomes by MALDI-TOF/MS

LCP and SK-Mel28 melanoma cell lines and exosomes have been analyzed by MALDI-TOF/MS using the intact method in order to perform statistical analysis of their lipid components.

Exosomes were purified from supernatants of SK-Mel28 and LCP cells at a final concentration of 0.2–1.1 $\times$ 10^{11} vesicles/ml. **Supplementary Figure 2** illustrates specific characteristics of exosomes, including the histogram that represents the size distribution of nanovesicles purified from LCP-conditioned supernatants (Panel A), TEM images showed the typical cup-shaped morphology of exosomes (Panel B), the presence of CD81, CD63, or CD9 tetraspanins (Panel C), and, finally, the western blot analyses (Panel D) confirm that exosome preparations were positive for typical markers of EVs (CD81 and TSG101), while excluded the possible contamination by large-EVs (>200 nm) or non-EV structures, such as protein aggregates, since all the samples resulted negative for both CANX and BSA as compared to the complete control medium.

MALDI Negative Ion Mode Analysis

Figure 3 shows the comparison between the representative lipid profiles of two melanoma cell lines, LCP and SK-Mel28 in Panels A and B, respectively, and those of exosomes they produce in Panels C and D, respectively, obtained by negative ion mode MALDI-TOF/MS analysis.

By comparing the four panels, it can be seen that, although not perfectly overlapping, the lipid profiles of the two cell samples (**Figures 3A,B**) share many similarities as well as no qualitative differences can be observed between the lipid patterns of the LCP-derived exosomes and SK-Mel28-derived exosomes (**Figures 3C,D**, respectively).

The main signals in the MALDI-TOF mass spectra, attributable to the negative $[M\text{-}H]^-$ molecular ions of all lipid classes here detected, are collected in **Table 1**.

The main peaks in both mass spectra of melanoma cells (**Figures 3A,B**) are visible in the range of major glycerophospholipids. The signals at *m*/*z* 835.6, 863.6, and 885.5 are assigned to various PI species. We also performed PSD analysis of the higher signal 885, which highlights the presence of an arachidonic acid (C20:4) and a stearic acid (C18:0) in the molecular structure of PI 38.4 (not shown). The peaks at *m*/*z* 716.5 and 744.6 could be attributable both to PE and PlsS species, while the peak at *m*/*z* 788.6 corresponds to PS. Other signals, attributable to various species of PI, PE, PA, SM, PS, and lysophosphatidylethanolamine (LPE) species, are also visible in both mass spectra as minor peaks (**Table 1**).

Additional peaks at *m*/*z* 1151.8, 1179.8, 1235.9, and 1261.9 can be observed in the *m*/*z* 1,100–1,500 enlargement of the mass spectra (**Figures 3A,B**, inset). These MALDI signals are compatible with complex acidic glycosphingolipids, carrying three sugar residues in their molecules and different fatty acids, named GM3. Furthermore, the minor peaks detected in the two lipid profiles of cells at *m*/*z* 1404.0 and 1430.0 correspond to the main CLs species (**Figures 3A,B**, inset).

As said before, the exosome lipid profiles in the negative ion mode of MALDI-TOF/MS are shown in the **Figures 3C,D**; both the lipid patterns are dominated by signals in the *m*/*z* range 600–800, compatible with major glycerophospholipid and sphingophospholipid species.

In the range of phospholipids, the major MALDI peaks are at *m*/*z* 687.6 and 797.7; the first signal is attributable to the sphingolipid SM, while the second one is attributable to the glycerophospholipid bis(monoacylglycero)phosphate (BMP).

The BMP molecules are similar to phosphatidylglycerol (PG) molecules in terms of mass, but the difference is in the bonding position of the two acyl chains: the two fatty acid chains are at *sn*-1 and *sn*-2 positions on the same glycerol backbone, in the PG structure; while, in the BMP structure, the two fatty acid chains are esterified at the sn-1 position of each glycerol (Holbrook et al., 1992; Hankin et al., 2015). This structural variation makes identification of PG and BMP lipid species *via* accurate mass alone almost impossible since they have the same exact molecular formula (Anderson et al., 2017).

Therefore, in order to elucidate the chemical identity of the high peak at *m*/*z* 797.7 in the mass spectra of exosomes, we have performed additional analyses. **Supplementary Figure 3** shows the TLC lipid profile of exosomes where individual phospholipids were identified by comparison of their R_f value with those of lipid standards; it consists mainly of (in R_f order) lysophosphatidylcholine (LPC), SM, PS, PC, PE, PA, and BMP, with PC as the most abundant phospholipid in the membrane of exosomes. CL, the phospholipid marker of mitochondria, was

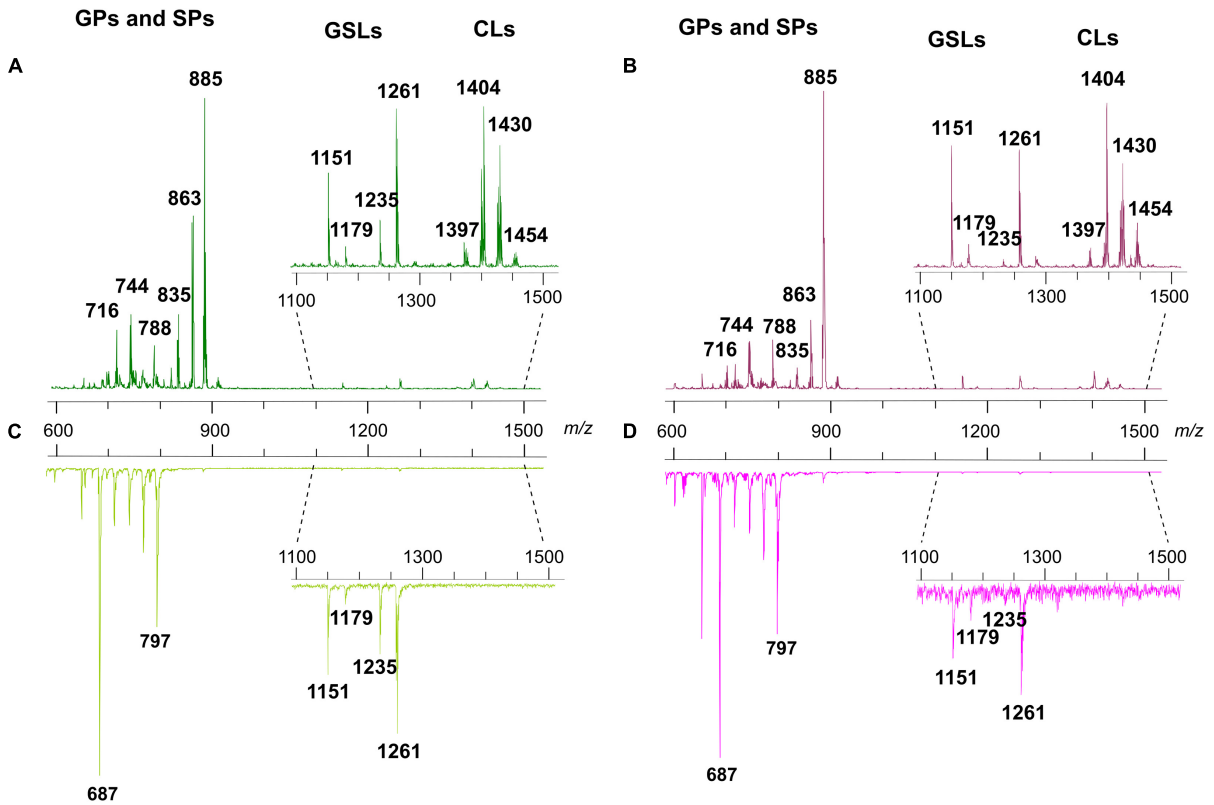

FIGURE 3 | Negative ion mode MALDI-TOF/MS lipid profiles of two melanoma cell lines and the derived exosomes. The upper spectra show the typical lipid profiles of LCP **(A)** and SK-Mel28 **(B)** cells by the intact method. The lower spectra show the typical lipid profiles of intact exosomes derived from LCP **(C)** and SK-Mel28 **(D)** cells. The enlargements of range *m/z* 1,100–1,500 of mass spectra are also shown. GPs, glycerophospholipids; SPs, sphingolipids; GSLs, glycosphingolipids; CLs, cardiolipins.

not found by TLC analysis, in agreement with data obtained by MALDI-TOF analysis of exosomes, where no signals attributable to CL species were visible (see **Figures 3C,D**, inset).

As regards BMP, it is noteworthy that the higher (in R_f order) lipid spot of exosomes, having the same R_f value of BMP 36:2 standard, was assigned to BMP; whereas, the R_f value of PG 36:2 standard is different due to the different positions of the acyl chains linked to the glycerol backbone (**Supplementary Figure 3**).

Furthermore, PSD analysis of the peak at *m/z* 797.7 (shown in **Supplementary Figure 4**) validates the chemical structure of BMP 38:4 constituted of stearic acid (C18:0) and arachidonic acid (C20:4) as fatty acid tails.

Finally, both the MALDI mass spectra of exosomes shown in **Figures 3C,D** present some signals corresponding to other glycerophospholipids species, such as PA, PE, PS, and PI, as minor peaks. Furthermore, smaller signals assigned to GM3 species are visible in the *m/z* 1,100–1,300 enlargement (see **Figures 3C,D**, inset).

Significant differences in intensity of MALDI signals detected in the negative ion mass spectra of melanoma cells (upper panels) and exosomes (lower panels) are reported as histograms in **Figures 4A–G**. First, by comparing series of replicates of LCP and SK-Mel28 cell lines mass spectra, we found that the following peaks at *m/z* 833.5, 835.6, 861.5, and 863.6, attributable to PI species 34:2, 34:1, 36:2, and 36:1, respectively, were significantly higher in the LCP sample (**Figure 4A** upper panel). Whereas the peak at *m/z* 885.5 (corresponding to PI 38:4) was significantly higher in SK-Mel28 than the LCP sample (**Figure 4A**, upper panel), suggesting that PI species with shorter acyl chains and having one and two double bonds are more abundant in the LCP than in the SK-Mel28 cell line.

No other significative differences in MALDI negative ion mode analyses between the two cell lines were observed, except an higher peak at *m/z* 716.5, corresponding to PE 34:1 (**Figure 4B**, upper panel) and PlsS P-32:1 in the LCP sample (**Figure 4C**, upper panel), and two higher peaks, at *m/z* 1151.8 and 1179.8, assigned to GM3 16:0 and 18:0, respectively, in the SK-Mel28 sample (**Figure 4D**, upper panel).

The MALDI peaks corresponding to the main PI, PE, and PS species (**Figures 4A–C**) present in exosomes mass spectra were significantly lower than those present in their parental cells. It is worth noting that only the signals corresponding to saturated and monounsaturated PE species (P-30:0/O-30:1, 34:0, and 38:1) were significantly higher in the exosomes than in their parental cells (**Figure 4B**). As regards GM3 species, the signals (at *m/z* 1151.8, 1261.9, and 1263.9) assigned to GM3 16:0, 24:1, and 24:0, respectively, were significantly lower in the MALDI lipid profile

TABLE 1 | Assignments of *m/z* values detected in negative ion mode of lipids mode MALDI-TOF mass spectra of lines and exosomes.

m/z value	Assignment $[M-H]^-$
478.3	LPE (18:1)
480.3	LPE (18:0)
506.3	LPE (20:1)
645.4	PA (32:1)
646.4	PlsE (P-30:0)/PlsE (O-30:1)
653.3	PlsA (P-34:3)/PlsA (O-34:4)
659.5	PlsA (P-34:0)/PlsA (O-34:1)
673.4	PA (34:1)
687.6	SM (16:0)
697.3	PA (36:3)
701.5	PA (36:1)
714.5	PE (34:2)
716.5	PE (34:1)/PlsS (P-32:1)
718.5	PE (34:0)
722.4	PE (P-36:4)
730.5	PS (32:2)
742.5	PE (36:2)
744.6	PE (36:1)/PlsS (P-34:1)/PlsS (O-34:2)
750.5	PlsE (P-38:4)
758.4	PS (34:2)
760.4	PS (34:1)
766.5	PE (38:4)
768.5	PE (38:3)
769.6	BMP (36:4)
770.5	PE (38:2)/PlsS (P-36:2)/PlsS (O-36:3)
771.7	BMP (36:3)
772.6	PE (38:1)
786.6	PS (36:2)
788.6	PS (36:1)
797.7	BMP (38:4)
799.7	BMP (38:3)
833.5	PI (34:2)
835.6	PI (34:1)
861.5	PI (36:2)
863.6	PI (36:1)
883.5	PI (38:5)
885.5	PI (38:4)
887.6	PI (38:3)
911.5	PI (40:5)
913.6	PI (40:4)
1151.8	GM3 (16:0)
1179.8	GM3 (18:0)
1235.9	GM3 (22:0)
1261.9	GM3 (24:1)
1263.9	GM3 (24:0)
1399.9	CL (68:4)
1404.0	CL (68:2)
1425.9	CL (70:5)
1430.0	CL (70:3)

The numbers (x:y) denote the total length (as carbon numbers) and number of double bonds of acyl chains, respectively.

TABLE 2 | Assignments of *m/z* values detected in positive ion mode MALDI-TOF mass spectra of melanoma cell lines and exosomes.

m/z value	Assignment $[M+H]^+$
494.4	LPC (16:1)
496.3	LPC (16:0)
522.3	LPC (18:1)
550.5	LPC (20:1)
703.5	SM (16:0)
706.5	PC (30:0)
720.6	PlsC (O-32:0)
725.5	SM (16:0) (+ Na^+)
732.5	PC (32:1)
734.5	PC (32:0)
742.5	PlsC (P-34:2)/PlsC (O-34:3)
746.6	PlsC (P-34:0)/PlsC (O-34:1)
758.5	PC (34:2)
760.5	PC (34:1)
786.5	PC (36:2)
788.6	PC (36:1)
810.5	PC (38:4)

The numbers (x:y) denote the total length (as carbon numbers) and number of double bonds of acyl chains, respectively.

of SK-Mel28-derived exosomes compared to those present in parental cells (**Figure 4D**); while no significant difference in GM3 signal intensities has been observed between the lipid profiles of LCP-derived exosomes and their parental cells (**Figure 4D**).

Furthermore, the MALDI peaks corresponding to PA P-34:3/O-34:4 and 34:1 (at *m/z* 653.3 and 673.4, respectively) (**Figure 4E**), SM 16:0 (at *m/z* 687.7) (**Figure 4F**) and BMP 36:3, 38:4, and 38:3 (at *m/z* 771.7, 797.7, and 799.7) (**Figure 4G**) were significantly higher in the lipid profiles of exosomes compared with those present in their parental cells mass spectra.

In conclusion, by comparing the two lipid profiles of melanoma cells with variable metastatic propensity, we observed more saturated and shorter fatty acid tails in poorly metastatic (LCP) cells compared with highly metastatic (SK-Mel28) cells, particularly for some species of PI, whereas the content of polyunsaturated PI 38:4 and GM3 species with saturated acyl chains increases in the SK-Mel28 cells.

Finally, no significant differences in the MALDI signals were observed between the lipid profiles of the two exosome preparations.

MALDI Positive Ion Mode Analysis

Figure 5 shows the comparison between the representative lipid profiles of the two melanoma cell lines LCP and SK-Mel28, in Panels A and B, respectively, and those of exosomes they produce in Panels C and D, respectively, obtained by positive ion MALDI-TOF/MS analyses; it can be seen that the higher peaks are present in the *m/z* range 600–800 of the lipid profiles, while smaller peaks are detectable in the lower *m/z* range 400–600. The main signals in the MALDI-TOF mass spectra, attributable to the positive $[M+H]^+$ molecular ions of glycerophospholipids and sphingophospholipids, are collected in **Table 2**.

In both the melanoma cells mass spectra (**Figures 5A,B**), the peaks at *m/z* 706.5, 732.5, 734.5, 760.5, and 786.5 attributable to the molecular ions $[M+H]^+$ of PC species are predominant. Furthermore, various plasmenylcholine (PlsC) species were barely detected as minor peaks. Smaller MALDI signals at *m/z*

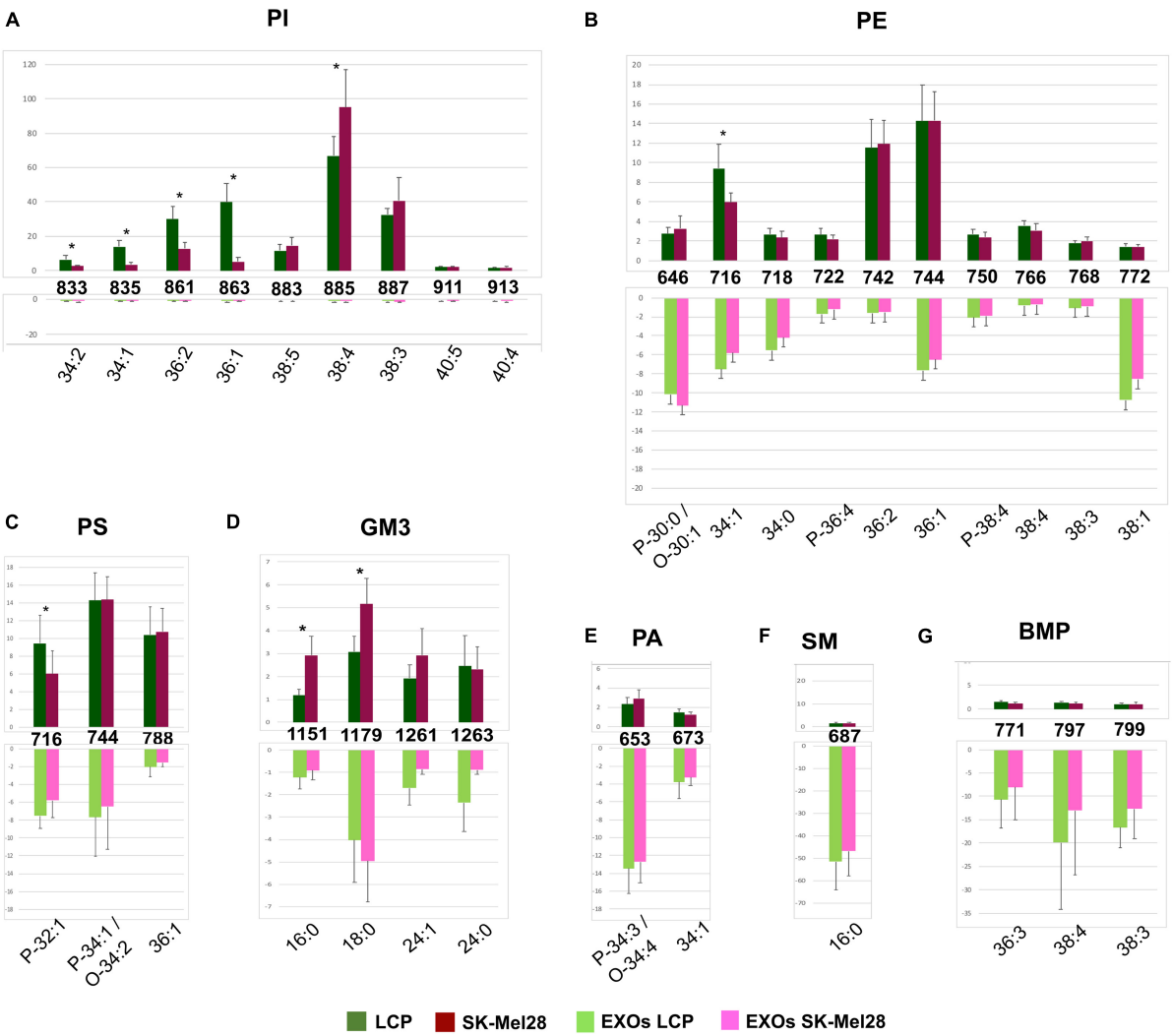

FIGURE 4 | Significant differences of (–) MALDI signals in lipid profiles of two cell lines (upper panels) and the derived exosomes (lower panels). The histograms **(A–G)** show the significant differences in intensity between the lipid peaks present in the four series of (–) mass spectra. *p*-value < 0.05 was set as the threshold to define significant differences. Significant differences between the signals detected in the cell lines profiles (shown in the upper panels) are also highlighted by an asterisk. Data are reported as the average value of intensity ± SD. The numbers reported between upper and lower panels indicate the MALDI *m/z* signals. Lipid assignments for each signal are also indicated.

496.3, 522.3 and 550.5 are visible in the enlargements of both the mass spectra, and are compatible with the molecular ion $[M+H]^+$ of LPC species (**Figures 5A,B**, inset).

In the lipid mass spectra of the two exosome preparations, shown in **Figures 5C,D**, the MALDI signals compatible with PC species are not dominant, while their intensities are similar to those of LPC species.

Furthermore, the molecular species corresponding to SM 16:0 was found in both the exosome lipid profiles as a signal at *m/z* 725.5, corresponding to the sodiated form of the molecule, also previously described by negative ion mode MALDI analysis (peak at *m/z* 687 in **Figures 3C,D**).

Figures 6A–C show significant differences in intensity of MALDI peaks detected in the positive ion mass spectra of melanoma cells (upper panels) and exosomes (lower panels).

First, by comparing series of replicates of LCP and SK-Mel28 cell lines mass spectra, we found that the following peaks were significantly higher in the LCP sample: PlsC O-32:0 and PlsC P-34:0/O-34:1 (at *m/z* 720.6 and 746.6, respectively) (**Figure 6A**, upper panel). No other significant differences in peak intensities were found between the lipid profiles of the two cell lines (see **Figures 6B,C**, upper panels).

As regards differences between exosomes and parental cells, the signal at *m/z* 742.5, assigned to the molecular ion $[M+H]^+$ of PlsC P-34:2/O-34:3, was significantly higher in the exosomes (**Figure 6A**); while those corresponding to PlsC O-32:0 and P-34:0/O-34:1 were significantly lower (see histograms in **Figure 6A**).

The signals at *m/z* 706.5, 734.5, 760.5, and 786.5, assigned to the molecular ions $[M+H]^+$ of PC 30:0, 32:0, 34:1, and

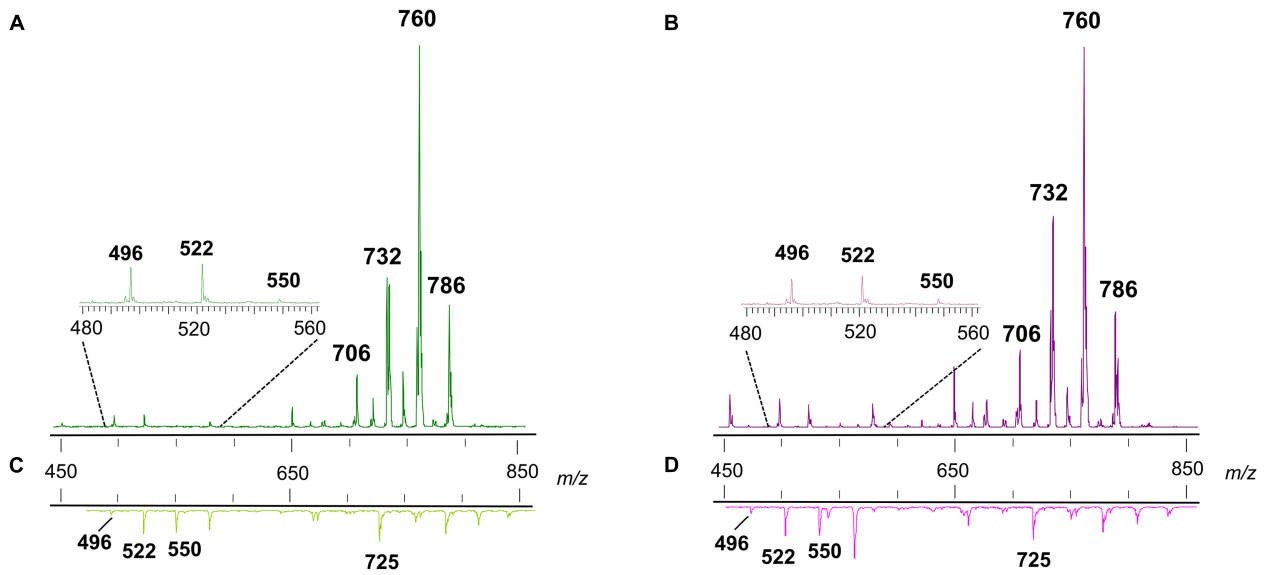

FIGURE 5 | Positive ion mode MALDI-TOF/MS lipid profiles of two melanoma cell lines and the derived exosomes. The upper spectra show the typical lipid profiles of LCP **(A)** and SK-Mel28 **(B)** cells by the intact method. The lower spectra show the typical lipid profiles of intact exosomes derived from LCP **(C)** and SK-Mel28 **(D)** cells. The enlargements of range *m/z* 480–560 of mass spectra are also shown.

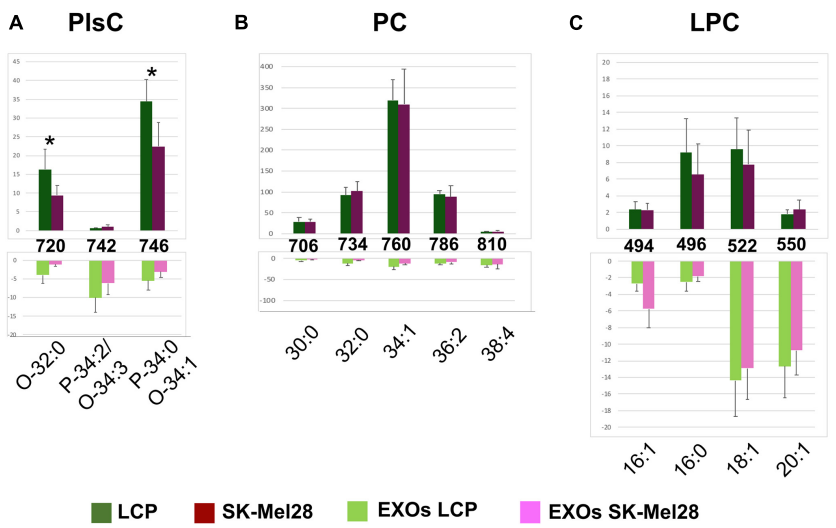

FIGURE 6 | Significant differences of (+) MALDI signals in lipid profiles of two cell lines (upper panels) and the derived exosomes (lower panels). The histograms **(A–C)** show the significant differences in intensity between the lipid peaks present in the four series of (+) mass spectra. *p*-value < 0.05 was set as the threshold to define significant differences. Significant differences between the signals detected in the cell lines profiles (shown in the upper panels) are also highlighted by an asterisk. Data are reported as the average value of intensity ± SD. The numbers reported between upper and lower panels indicate the MALDI *m/z* signals. Lipid assignments for each signal are also indicated.

36:2 species, respectively, were significantly higher in the lipid profiles of melanoma cells, being very lower in those of exosomes. Only the signal corresponding to PC 38:4 (at *m/z* 810.5) was significantly higher in the exosomes than in the parental cells (see histograms in **Figure 6B**).

Regarding the LPC species, only the peak at *m/z* 496.3, corresponding to saturated species LPC 16:0, was significantly lower in the exosomes than in their parental cells, while the peaks at *m/z* 494.4, 522.3, and 550.5, assigned to the monounsaturated species LPC 16:1, 18:1, and 20:1, respectively, were significantly higher in exosomes (see histograms in **Figure 6C**).

In conclusion, poorly metastatic cells contain higher levels of PlsC than highly metastatic ones; as regards to both exosomes, a decrease of PC and an increase of LPC content were observed in comparison to parental cells.

DISCUSSION

The present article is the first lipidomic-based study that characterizes exosomes derived from melanoma cell lines, having different metastatic potential, as previously characterized (Mannavola et al., 2019, 2020).

Although the MALDI-TOF/MS technique is not suitable for analyzing the fatty acids content in a biological sample, our results show that, in general, melanoma cells contain low levels of polyunsaturated phospholipid species, having fatty acid chains mainly constituted of 16 and 18 carbon atoms. Furthermore, the detected sphingolipid species, such as SM and GM3, were mainly constituted of saturated fatty acids (palmitic and stearic acids).

It is well-known that different chain lengths and degrees of unsaturation are able to affect the physical properties of cell membrane, either modifying its fluidity or stabilizing membrane proteins in the lipid domains (Szlasa et al., 2020). The question whether cell membrane rigidity could affect proliferation or metastasis of cancer cells has not yet been answered. Recently, it has been reported that the membrane fluidity and plasticity increased in cancer cells to facilitate their penetration into the blood vessels (Szlasa et al., 2020), and that a fluid membrane accelerates cell adhesion and metastatic capacity, for example, in breast cancer (Ross et al., 2016).

The differences of the composition in fatty acids between melanoma cells, having different metastatic potentials, and, in particular, the reduction of the degree of unsaturation and the length of the acyl chains in poorly metastatic cells here found, agree with previous reports describing melanoma cells with a reduced tumor cell migration, containing saturated phospholipids (Ross et al., 2016).

As regards PI, a progressive increase in some species with saturated and monounsaturated fatty acyl chains was associated with melanoma metastasis and progression, and these lipid species can be considered novel biomarkers for estimating the metastatic ability of melanoma cells (Kim et al., 2017). Although our findings on the higher level of PI 38:4 (containing both arachidonic and stearic acid) in highly metastatic melanoma cells disagree with the previous evidence, it can be also considered that a higher content of polyunsaturated fatty acids increases the membrane fluidity of these cells.

The higher content of C20:4 in highly metastatic melanoma cells could correlate with progression and metastatic behavior cells. In general, phospholipid species containing C20:4 can be hydrolyzed by phospholipase A2, and the produced C20:4 is converted into prostaglandin E2 (PGE2) by cyclooxygenase-2 and PGE synthase (Wang and DuBois, 2010). It is noteworthy that the levels of these species containing C20:4 were decreased in melanoma cells compared with those in normal cells and were associated with increase in PGE2, which contributes to the development, progression, and metastasis of cells (Kim et al., 2017).

As regards the content of the main glycosphingolipid detected, the enrichment in GM3 containing saturated fatty acids in the highly metastatic melanoma cells is in contrast with its general suppressive effect on cancer development and progression (Hakomori and Handa, 2015). GM3 is highly enriched in a type of membrane microdomain termed "glycosynapse" and forms complexes with co-localized cell-signaling molecules, including certain proteins tetraspanins enriched in exosomes membranes. GM3 modulates cell adhesion, growth, and motility by altering molecular organization in glycosynaptic microdomains and the activation levels of co-localized-signaling molecules that are involved in cancer pathogenesis (Hakomori and Handa, 2015). Moreover, it has been reported that GM3 downregulates the invasiveness capacity of human bladder cancer cells, while it can prevent haptotactic cell migration in colorectal cancer cell lines (Palacios-Ferrer et al., 2021). Further studies will be necessary to elucidate possible roles of this lipid in metastasis progression of melanoma.

Another interesting aspect concerns plasmalogens, which represent up to 20% of the total phospholipid mass in humans and appear to be associated with common disorders and diseases, including cancer (Braverman and Moser, 2012; Messias et al., 2018). In general, cancer cells are enriched in alkyl and alk-1-enyl ether lipids compared with normal cells, enough to be considered as a potential diagnostic marker for some species of cancer (Fernandes et al., 2020). Thanks to our MALDI-TOF lipid analyses, we detected various Pls species (such as PlsE, PlsS, PlsA, and PlsC) in both melanoma cell lines; noteworthy is the significant decreased content of PlsC O-32:0 and PlsC P-34:0/O-34:1 species in the highly metastatic cells. We can speculate that, in particular, the lower content of PlsC could be related to high oxidative stress associated with cancer progression, according to previous evidence on ovarian cancer cells, where decreased PlsC levels have been reported and correlated with oxidative stress (Hou et al., 2016).

As it was previously reported that LCP-derived exosomes were able to induce osteotropism in SK-Mel28 cells (Mannavola et al., 2019), we have also investigated if the quantitative differences of the lipid species observed between the two cell lines matched with any differences in the exosomes they produce.

It is well-known that exosomes carry bioactive lipids, which trigger cell-to-cell signaling, but the lipid-related aspects of exosomes have not obtained sufficient attention in the scientific literature.

Some studies have shown an enrichment of lipid species, including CHOL, SM, glycosphingolipids, and PS in exosome membranes; in contrast, exosomes generally contained lower levels of PC and PI than their parental cells, and only small changes were reported for PE content (Baenke et al., 2013; Record et al., 2014; Lydic et al., 2016; Skotland et al., 2017, 2019). This change in lipid composition apparently increases the exosomes ability to fuse with neighboring cells (Tickner et al., 2014). Lipidomics data appear to be inconsistent, and there is poor knowledge about lipid profiling of exosomes from melanoma cells.

These data are the first lipidomic analysis of exosomes derived from LCP and SK-Mel28 melanoma cells by MALDI-TOF/MS in positive and negative ion modes. No significant differences between the two lipid profiles of exosomes are observed in our experiments, but, as expected, the intensities of some signals of lipids in the mass spectra of exosomes are very different from those of their parental cells.

In agreement with previous reports, our statistical analyses of the MALDI lipid profiles of both exosome preparations clearly show an enrichment in the sphingolipid SM, but also in other glycerophospholipid species, such as PA, LPC, and BMP, compared with their parental cells. It is worth noting that the levels of polyunsaturated species of PC and PlsC were significantly higher in the exosomes; as previously indicated, the high content of double bonds in the membrane lipids makes the exosome membrane more fluid, which is a fundamental characteristic in the fusion process of vesicles with neighboring cells. Besides, only few signals corresponding to PE species were significantly higher in the exosomes than in their parental cells. Furthermore, we show a clear decrease of the content of PI species in the two exosomes preparations compared to their parental cells. Also, other MALDI-TOF/MS signals corresponding to phospholipids PE, PS, PC, and PlsC species were significantly lower in exosomes.

A higher content in SM, PA, and LPC in exosome membranes compared to parental cells highlights the presence of membrane microdomains necessary for cell-cell communication and cell-signaling functions. The high level of LPC in exosomes could depend on its intracellular origin (i.e., endolysosomal compartment), but it can be also related to the role of this lysocompound as substrate for autotaxin, which is a lysophospholipase D that converts LPC in LPA (Moolenaar and Perrakis, 2011). It is known that autotaxin, to which exosomes can bind, is involved in the motility stimulation and has been also found in melanoma cells (Jethwa et al., 2016). It has been also suggested that the extracellular hydrolysis of phospholipids like LPC by metastatic tumor cells and the subsequent cellular uptake of the resulting free fatty acids seem to be a necessary prerequisite for metastatic potential of epithelial tumor cells, probably for generating pro-metastatic lipid second messengers (Raynor et al., 2015).

Moreover, it is interesting the intriguing presence of the polyglycerophospholipid BMP in exosomal membranes, since its presence in the vesicles is still controversial in the litterature. Its unusual lipid structure consists of two monoacylglycerols linked through one phosphate group; it is found in most mammalian cells and tissues, where it represents only 1% of cellular phospholipids, but it was found to be mostly enriched in lysosomes and endosomes. However, its presence in the exosomes is still unclear. This derives from the complexity of its identification that mostly depends by its modest content as well as the difficult discrimination from PG. Some authors have hypothesized that the amount and the distribution of BMP are cell type dependent, and/or BMP-enriched exosomes can be released under endolysosomal stress (Jabs et al., 2008; Miranda et al., 2018; Rabia et al., 2020).

To our knowledge, there are poor data on the presence of BMP in tumor cell-derived exosomes and, in particular, in those from melanoma. The combined lipid analysis by TLC and MALDI-TOF/MS allowed us to confirm the presence of various BMP species in melanoma exosomes, and statistical analysis leads us to conclude that it is significantly enriched compared with their parental cells. It can be added that the BMP enrichment in exosomes may be related to the biogenesis process rather than to its possible role in cancer progression.

Although we observed significant differences in some lipid species potentially implicated in the variable osteotropic propensity between the analyzed melanoma cells, we did not find any quantitative difference between the lipid components of the two exosome preparations.

To this regard, the limited sample size may have affected our experimental results. Further investigation, including *in vivo* isolation of exosomes, as well as parental tumor cells from either metastatic or not-metastatic melanoma patients, should be addressed to improve our lipid analysis.

On the other hand, we cannot exclude a biological reason for not identifying potential lipid biomarkers for different osteotropic behaviors of melanoma-derived exosomes. Has metastatic potential no influence on lipid sorting into exosomes? Do Exosomal lipids not mediate metastatic potential? Additional studies and analyses will be necessary to answer these crucial questions.

In conclusion, here, we used the direct MALDI-TOF/MS analysis, an extremely sensitive analytical technique, to achieve the most comprehensive lipid analysis of melanoma exosomes reported to date. Therefore, our data make a useful contribution to a comprehensive understanding of melanoma-secreted exosomes lipid molecular compositions. Understanding the precise physiological function of exosomes will be critical to determining their important role in cancer.

The basic knowledge of lipid species (quality and relative abundance) of the cell membranes (and in specific intracellular compartments) of melanoma cells might help in developing new pharmacological approaches to affect cell survival and reduce bone metastasis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AC and FS designed the research. FM and MT provided samples. CF, PL, CI, FM, and PT performed the research. AC, SL, MT, and PL analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.748895/full#supplementary-material

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The Crucial Roles of Phospholipids in Aging and Lifespan Regulation

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Phospholipids are major membrane lipids that consist of lipid bilayers. This basic cellular structure acts as a barrier to protect the cell against various environmental insults and more importantly, enables multiple cellular processes to occur in subcellular compartments. Numerous studies have linked the complexity of membrane lipids to signal transductions, organelle functions, as well as physiological processes, and human diseases. Recently, crucial roles for membrane lipids in the aging process are beginning to emerge. In this study, we summarized current advances in our understanding of the relationship between membrane lipids and aging with an emphasis on phospholipid species. We surveyed how major phospholipid species change with age in different organisms and tissues, and some common patterns of membrane lipid change during aging were proposed. Further, the functions of different phospholipid molecules in regulating healthspan and lifespan, as well as their potential mechanisms of action, were also discussed.

Keywords: membrane lipid, aging, phospholipid, lifespan, organelle

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INTRODUCTION

The relationship between lipids and aging has been well recognized. The contents, composition, and metabolism of fatty acid (FA) are altered in aged or long-lived humans and model organisms (Papsdorf and Brunet, 2019). Moreover, studies in model organisms such as *Caenorhabditis elegans* have revealed that various FA species could extend lifespan when supplemented in the diet, which includes monosaturated oleic acid, palmitoleic acid, cis-vaccenic acid, and oleoylethanolamine, as well as polyunsaturated a-linolenic acid, arachidonic acid, and dihomo-g-linolenic acid (Goudeau et al., 2011; Rourke et al., 2013; Folick et al., 2015; Han et al., 2017; Qi et al., 2017). These unsaturated FAs function mainly through classic longevity factors, such as DAF-16/FOXO3, SKN-1/Nrf2, and HSF-1/HSF1, to regulate healthspan and lifespan (Labbadia et al., 2015; Steinbaugh et al., 2015; Papsdorf and Brunet, 2019).

Despite these advances linking FA to longevity regulation, little is known about their mechanisms of action. Generally, FAs function through several major mechanisms, including signaling molecules, energy resources, substrates for post-translational modifications, and the components of complex lipids (Van Meer et al., 2008; Shimizu, 2009; Nakamura et al., 2014; Saliba et al., 2015; Resh, 2016; Harayama and Riezman, 2018). Take oleoylethanolamine for example, it acts as a signaling molecule and regulates animal lifespan by direct binding and activation of the nuclear hormone receptor NHR-80 (Folick et al., 2015). But to date, only a few FAs were found to exert their functions directly as signaling molecules, or as substrates for post-translational modifications.

The majority of FAs are incorporated into complex lipids such as membrane lipids as their acyl chains, thus affecting the structure, composition, and function of the membrane (Van Meer et al., 2008; Sezgin et al., 2017; Harayama and Riezman, 2018). Therefore, it is conceivable that FAs may regulate lifespan by acting as the important components of membrane lipids, potentially linking membrane homeostasis to lifespan regulation.

Membrane lipids, mainly phospholipids (PLs; also known as glycerophospholipids), consist of the lipid bilayer that acts as barriers between the cell and environment, and between different cellular compartments. However, numerous studies suggest that the lipid bilayer not only function as structural barriers but also play crucial roles in the regulation of multiple cellular processes (Shimizu, 2009; Wu et al., 2016; Sunshine and Iruela-Arispe, 2017; Harayama and Riezman, 2018). Also, this idea is supported by the diversity of membrane lipids (different membrane lipid species and different acyl chains within certain membrane lipids) (Hishikawa et al., 2014; Antonny et al., 2015), which is far more beyond the need for barrier function. In regard to the aging process, studies in several model organisms have reported the association of the contents and compositions of many membrane lipids with animal age (Papsdorf and Brunet, 2019), supporting potential roles for the membrane lipids in aging modulation. In this review, we focused on PLs and summarized recent advances that link PL homeostasis to the aging process and discussed their potential mechanisms of action. Other membrane lipids such as sphingolipids were not discussed in the current review.

PHOSPHOLIPID METABOLISM AND DIVERSITY

Phospholipids are major structural lipids of the eukaryotic membrane, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and cardiolipin (CL). These major PL species share similar structures containing two FAs attached to the *sn*-1 and *sn*-2 positions and a different phosphate headgroup at the *sn*-3 position of the glycerol backbone. The different compositions of these PLs may account for the membrane diversity of various subcellular compartments and thus the functions of the organelles. Take PC as an example, it is the most abundant PLs that account for over 50% of total PLs in the cell. It mainly resides in the endoplasmic reticulum (ER), the site of membrane lipid biosynthesis, but its contents are relatively less in the plasma membrane (Van Meer et al., 2008). The maintenance of PC homeostasis is critical for the organellar function, while the reduction of PC represents cellular stress known as lipid bilayer stress (Volmer et al., 2013; Halbleib et al., 2017; Shyu et al., 2019). Therefore, the cell evolves an elegant adaptive mechanism to survey the PC content and the loss of PC has been found to affect multiple cellular processes through this stress-responsive pathway (Koh et al., 2018; Ho et al., 2020). Another extreme example of the diversity of PL composition is organelle-specific PLs. CL is a mitochondria-specific membrane lipid, whose content has been found to have great impacts on mitochondrial functions and to be related to various mitochondrial diseases (Chicco and Sparagna, 2007; Houtkooper and Vaz, 2008; Schlame, 2008; Pizzuto and Pelegrin, 2020).

The acyl chain composition also accounts for the diversity of PLs, which differ largely in the lengths of the chains and the numbers and positions of the double bonds. These chemical variations may affect the membrane protein-lipid interactions and therefore the cellular signaling properties of the membrane proteins (Antonny et al., 2015; Saliba et al., 2015; Wu et al., 2016; Harayama and Riezman, 2018). In addition, PLs containing the unsaturated acyl chains are more fluidic than the saturated ones and thus the overall degree of the FA unsaturation in PLs could affect the membrane fluidity, which has been found to regulate numerous signaling pathways, cellular processes, and human diseases (Los and Murata, 2004; D'Auria and Bongarzone, 2016; Ammendolia et al., 2021). Therefore, as for the aging study, it is important to understand how the contents and compositions of PLs interact with proteins in longevity pathways and how such interactions determine the healthspan and lifespan. In the following section, we will discuss the relationship between aging and essential PLs.

PHOSPHATIDYLCHOLINE

Association Between Phosphatidylcholine and Age

Multiple studies have reported the changes of PC contents as animal ages, with species and tissue specificity. For example, it was reported that the contents of most PCs are notably decreased in the aged nematodes (Gao et al., 2017; Wan et al., 2019). Additionally, the overall PC contents show a significant reduction in the kidney of aged mouse (Braun et al., 2016), while the accumulation of PCs has been detected in the hippocampus of a brain aging mice model (Lin et al., 2016). In line with this, major mitochondrial PC species are found to be increased in the brain of aged humans (Hancock et al., 2015, 2017). Thus, the reduction of overall PC contents appears to be a general feature of animal aging, except for the aged brain where the PC contents seem to be increased. Studies in long-lived organisms further support this idea, as overall PC contents are greater in multiple tissues of the long-lived naked mole-rat compared with that of mice (Mitchell et al., 2007). It is also the case for humans, as most PC species are higher in the centenarians than that in the elderly (Montoliu et al., 2014).

More intriguingly, besides the accumulation of overall PC contents, the long-lived centenarians have a unique metabolic signature of PC, showing that several specific PC species are unexpectedly decreased (Collino et al., 2013). These decreased PCs remain similar between the centenarians and young people, while elevating in the elderly (Collino et al., 2013), thus may representing a youth or longevity signature. A study in model organism *C. elegans* also shows that certain PCs, mainly some unsaturated ones, remain at low levels in long-lived mutants regardless of age (Wan et al., 2019). The long-lived naked mole rats also have low levels of PCs that contain unsaturated docosahexaenoic acid (DHA, 22:6) as acyl chains, compared with

mice. As such, we propose that specific PCs, most likely some unsaturated ones, are negatively associated with animal longevity.

Lifespan Regulation by Phosphatidylcholine

It seems that the overall PC contents and specific PCs are associated with the age difference. In *C. elegans*, a Dietary supplement of PC mixture can significantly extend the lifespan and improve the healthspan of nematodes via the longevity transcription factor DAF-16 (Kim et al., 2019), which is consistent with the idea that the decline of total PCs is not only a biomarker of aging but also a reason for it. The mechanism underlying the total PCs effects on lifespan is currently not clear but may be related to membrane fluidity. The decline of total PCs can cause membrane stiffening (Dawaliby et al., 2016) that is observed in aged cells and animals (Yu et al., 1992; Levi et al., 1997). Moreover, this age-associated decline of membrane fluidity can be reversed by longevity-inducing interventions like dietary restriction (Yu et al., 1992).

Unsaturated PC species may act differently with total PCs in the context of lifespan regulation. A recent *C. elegans* study reported that the levels of unsaturated PCs are decreased in a long-lived *C. elegans* model. More importantly, the reduction of unsaturated PC species likely function in the endoplasmic reticulum (ER) to promote longevity by activating the ER-resident calcium channel, downstream calcium-sensitive kinases, and the transcription factor DAF-16 (He et al., 2021), suggesting that specific unsaturated PCs may interact with membrane proteins to regulate longevity. The finding that reduction of unsaturated PC is beneficial for lifespan extension is consistent with the idea that low contents of unsaturated PCs may be a longevity signature. Understanding how the changes of certain unsaturated PCs determine lifespan deserves further investigation.

Phosphatidylcholine and Brain Aging

Aging is a complex process associated with the functional decline of essential organs and tissues, thus representing a major risk factor for many chronic diseases referred to as aging-related diseases. As PCs accumulate in the brain of aged animals (Hancock et al., 2015, 2017), studies also pay attention to the function of PCs in neurodegenerative diseases such as Alzheimer's disease (AD). In patients with AD, the contents of unsaturated PC 36:5 and PC 38:6 are lower in the plasma and are correlated with hippocampus atrophy (Kim et al., 2017). Consistently, the higher contents of several unsaturated PCs are associated with a slower decline in AD-related cognition (Li et al., 2019). By contrast, the accumulation of PC-O (16:0/2:0) is found to associate with multiple AD pathologies, such as the aggravated tau pathology, enhanced vesicular release, and signaling neuronal loss (Bennett et al., 2013). Thus, it appears that certain unsaturated PCs are critical for the functional maintenance of neurons.

It is not fully understood how certain PCs maintain neuronal function and protect against neurodegenerative diseases. A key and common pathology of neurodegenerative diseases is protein aggregation. The ER is critical for protein folding. when misfolded proteins accumulate in the ER (also known as ER stress), ER unfolded protein response is induced to assist protein folding and maintain cellular proteostasis. Indeed, PC disturbance has been linked to abnormal ER function and protein aggregation (O'Leary et al., 2018; Kumar et al., 2019; Shyu et al., 2019). As a consequence, PC disturbance is recognized as a stress to the ER and can promote the ER UPR (Volmer et al., 2013; Halbleib et al., 2017; Ho et al., 2020). A recent study of *C. elegans* also supports the crucial of certain PCs in ER homeostasis. The contents of unsaturated PC are increased in the somatic cells of *C. elegans* upon DNA damage, which represents an aging risk factor. Moreover, the elevation of unsaturated PCs is critical for promoting the ER UPR without causing ER

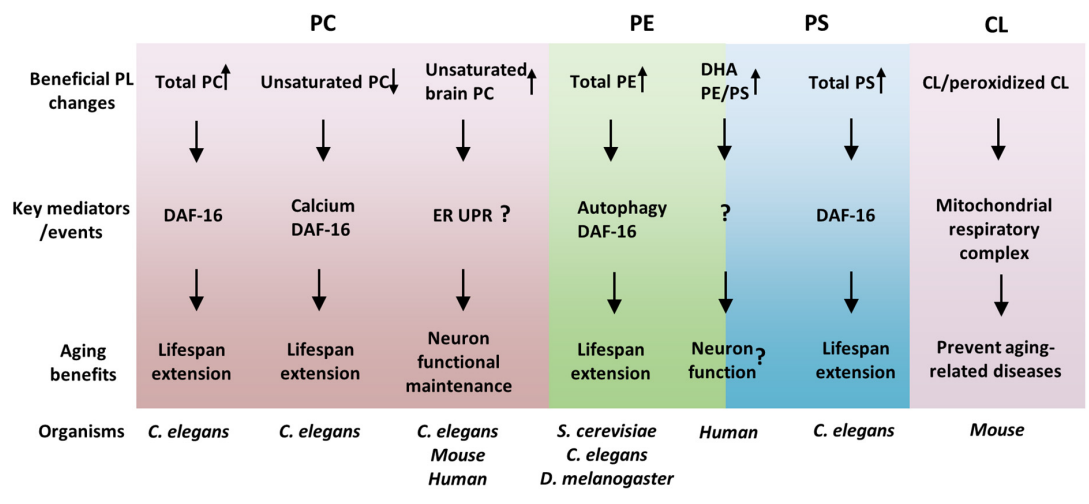

FIGURE 1 | Phospholipids (PLs) regulate aging *via* overlapped but distinct molecular mechanisms. PL species with beneficial effects on healthspan and lifespan were suggested and their key mediators were listed. Among the mediators, DAF-16 seems to be common and important for many PLs effects. Moreover, the functions of organelles, such as the ER and mitochondria, are crucial links between PLs and aging, consistent with PLs as membrane lipids.

stress, thus enhancing cellular maintenance (Deng et al., 2021). Intriguingly, the neurons and *C. elegans* somatic cells share some common features, as they are both postmitotic cells that cannot proliferate and are irreplaceable. In this regard, we propose that certain unsaturated PC species may be of great importance for the functional maintenance of postmitotic cells such as neurons during the aging process.

PHOSPHATIDYLETHANOLAMINE

Association Between Phosphatidylethanolamine and Age

Phosphatidylethanolamine is the second abundant PL in organisms. Emerging pieces of evidence show that PE contents also change with age. Major PE species decrease significantly in the aged nematodes (Gao et al., 2017), as well as in the brain and kidney of old mice (Braun et al., 2016; Lin et al., 2016), suggesting that PE reduction may also be a general feature of aging. Moreover, a human lipidomic study has found that the contents of ether lipid derived from PE are lower in the centenarians compared with that in the elderly people (Pradas et al., 2019), suggesting this specific form of PE may function as a centenarian signature that may promote longevity. Interestingly, although the contents of overall mitochondrial PE in the brains of the elderly are reduced, mitochondrial PEs containing unsaturated docosahexaenoic acid (DHA, 22:6) were found to be increased significantly (Hancock et al., 2015, 2017). Due to the importance of DHA for neuronal development and function, the increase in DHA-containing PEs with age may be an adaptive and protective mechanism for brain aging, suggesting specific PE species and overall PE contents may affect health during aging differentially.

Lifespan Regulation by Phosphatidylethanolamine

Studies has shown that the increase of PE contents, either by dietary supplementation of PE precursor ethanolamine or by the overexpression of PE-biosynthetic enzymes phosphatidylserine decarboxylases (PSD), extends lifespan in model organisms of yeast, fly, and mammals (Rockenfeller et al., 2015). And the lifespan extension effects of PE are associated with increased autophagic flux (Rockenfeller et al., 2015), a positive lifespan regulator in many model organisms (Hansen et al., 2018), suggesting PE supplementation may increase lifespan by promoting autophagy.

In contrast, the reduction of PE by suppressing PSD causes ROS production and accelerates aging in yeast (Rockenfeller et al., 2015). The relationship between PE and ROS is also supported by a *C. elegans* study that supplementation of PE enhances the resistance to oxidative stress and promotes longevity via DAF-16 (Park et al., 2021). These findings suggest that PE plays pivotal roles in life extension, likely acting as a regulator of ROS production. ROS is a byproduct of mitochondrial respiration and is generally associated with mitochondrial defects or dysfunction. Indeed, PSD-mediated PE production occurs in the mitochondrial inner membrane and is found to be essential for the activity of the electron transport chain (Calzada et al., 2019). Accordingly, PSD knockdown compromised the mitochondrial integrity and muscle mass in the mice (Selathurai et al., 2019). Thus, it is conceivable that mitochondria activity is key for lifespan determination in response to PE alteration.

PHOSPHATIDYLSERINE

The contents of major PS species are also found to decrease during aging (Lin et al., 2016; Šmidák et al., 2017). Consistently, supplementation of PS shows beneficial effects on lifespan and aging-associated pathologies. External supplement of PS could increase the oxidative stress resistance and extend the lifespan of nematodes via DAF-16 (Kim and Park, 2020). PS supplement could also improve the memory impairment of the old rodents as well as the human being (Vakhapova et al., 2010; Lee et al., 2015), which is consistent with a crucial role for PS in driving the functional recovery of severed axon and damaged neurons (Hulbert et al., 2007). Moreover, it was shown that compared with elderly unimpaired mice, PS contents in elderly mice with impaired memory are significantly reduced (Wackerlig et al., 2020), further suggesting a strong causal link between the PS contents and the decline of neuronal function during aging. Notably, the principal PS (18:0/22:6) is remarkably increased in the brains of elderly people, consistent with the beneficial role of DHA (22:6) on cognitive function. Both PE and PS containing DHA as acyl chains increase with age (Hancock et al., 2015, 2017; Norris et al., 2015), suggesting that DHA may protect against aging-related neuronal impairment via these PL species.

CARDIOLIPIN

Cardiolipin and Aging

Cardiolipin is a symbolic phospholipid of the mitochondrial membrane. CL plays a critical role in many aspects of the mitochondrial function: stabilizes respiratory chain supercomplex, regulates the activity of mitochondrial membrane proteins, and controls essential mitochondrial signaling pathways (Pfeiffer et al., 2003; Dudek, 2017). Due to the special location, CL is closely related to respiration as well as a normal function of the respiratory chain complex, which leads to its association with aging and age-related disease (Shi, 2010; Paradies et al., 2011; Hsu and Shi, 2017). Consistently, CL contents were found to be decreased in the aged nematodes and rodents (Gao et al., 2017; Šmidák et al., 2017), while the contents of CL are notably increased in the adipose tissues of long-lived model Ames dwarf mice compared with normal mice (Darcy et al., 2020), suggesting the idea that increased CLs are beneficial for health and longevity. Indeed, a yeast study shows that perturbation of CL synthesis leads to decreased longevity (Zhou et al., 2009).

Cardiolipin Peroxidation and Aging

Notably, CLs are highly sensitive to oxidative stress and the peroxidation of CLs can cause damage to the respiratory

chain complex, which in turn causes mitochondrial defects and respiratory dysfunction. In line with this, the contents of total CLs were found to decrease, while the peroxidized CLs are significantly increased in the brains of aged rats, which is associated with impaired activity of brain mitochondrial respiratory complex I (Petrosillo et al., 2008). The peroxidation of CLs is influenced by the CL remodeling, a process catalyzed by the acyltransferase (ALCAT1) that synthesizes CLs from lysocardiolipin (Cao et al., 2004). The resynthesized CLs are highly sensitive to oxidative damage by ROS due to the enriched double bonds in poly-unsaturated acyl chains. Thus, the pathological CL remodeling can exacerbate oxidative stress as well as mitochondrial dysfunction (Li et al., 2010, 2012), while the inhibition of CL remodeling may improve the aging-associated functional decline of mitochondria. Indeed, ALCAT1 inhibition or deficiency could improve mitochondrial function, alleviate oxidative stress and prevent disease progression in the context of many aging-related diseases including obesity, Parkinson's disease, aging-related heart disease, and non-alcoholic fatty liver disease (Li et al., 2010; Liu et al., 2012; Wang et al., 2015; Song et al., 2019). These findings link ALCAT1 to mitochondrial dysfunction and reveal a critical role for CL remodeling in age-related diseases. A balance between the non-peroxidized CLs and peroxidized CLs is crucial for maintaining mitochondrial function and enabling healthy aging.

CONCLUSION AND PERSPECTIVE

As reviewed here, the association between membrane PLs and aging, and the regulation of the aging process and aging-related diseases by PLs have been greatly advanced in recent years. PLs, regulate lifespan and healthspan via overlapped but different cellular and molecular mechanisms (**Figure 1**). Identification of particular PL species that are associated with lifespan modulation has expanded our knowledge of how metabolic reprogramming affects aging. Nevertheless, these new pieces of knowledge also raise many important and interesting questions, which include, but are not limited to, the following ones: (1) Why are the overall PLs generally decreased with age? It appears that the overall PLs regulate lifespan in ways different from the specific lipids. Whether PLs regulate the aging process via common mediators such as DAF-16/FOXO? If so, how does DAF-16 receive signals from multiple membrane PLs? (2) What are the mechanisms of lifespan regulation by specific PLs, such as unsaturated PCs? It is important to investigate what subcellular compartments the specific PLs function on and what membrane proteins these PLs interact with. (3) How are the contents of PLs regulated during aging? Are there any key transcription factors that control the expressions of PL metabolic enzymes during aging? (4) How does the cell control the homeostasis of PLs? It is unclear how does the cell sense the changes of PL contents and responds/adapts to such metabolic stresses.

The answers to these questions will provide deeper understandings of the function of PL homeostasis in lifespan and healthspan regulation. As the conservation of lipid metabolism across phyla and unique advantages of model organisms in an aging study (such as the short lifespan of *C. elegans* and the tissue similarity to human of rodents), studies combining different model organisms will together help to elucidate the crucial roles and mechanisms of PLs in regulating lifespan and aging-associated diseases.

AUTHOR CONTRIBUTIONS

SP conceived the concept of this manuscript. YD, HT, and SP analyzed literatures related to this review topic and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Lipid Droplets, the Central Hub Integrating Cell Metabolism and the Immune System

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Lipid droplets (LDs) are commonly found in various biological cells and are organelles related to cell metabolism. LDs, the number and size of which are heterogeneous across cell type, are primarily composed of polar lipids and proteins on the surface with neutral lipids in the core. Neutral lipids stored in LDs can be degraded by lipolysis and lipophagocytosis, which are regulated by various proteins. The process of LD formation can be summarized in four steps. In addition to energy production, LDs play an extremely pivotal role in a variety of physiological and pathological processes, such as endoplasmic reticulum stress, lipid toxicity, storage of fat-soluble vitamins, regulation of oxidative stress, and reprogramming of cell metabolism. Interestingly, LDs, the hub of integration between metabolism and the immune system, are involved in antitumor immunity, anti-infective immunity (viruses, bacteria, parasites, etc.) and some metabolic immune diseases. Herein, we summarize the role of LDs in several major immune cells as elucidated in recent years, including T cells, dendritic cells, macrophages, mast cells, and neutrophils. Additionally, we analyze the role of the interaction between LDs and immune cells in two typical metabolic immune diseases: atherosclerosis and Mycobacterium tuberculosis infection.

Keywords: lipid droplets, immune cells, atherosclerosis, metabolism, immunity

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INTRODUCTION

Lipid Droplets

Lipid droplets (LDs), organelles related to cell metabolism that store lipids in different cells, vary greatly in number, size, and structure. LDs consist of the core of the neutral lipid surrounded by a polar lipid monolayer with the polar head group of phospholipids facing the cytosol their and acyl chains contacting the hydrophobic neutral lipid nucleus, while the surface of the LDs contains many different proteins that are related to the specific function of the LDs (Kory et al., 2016). Commonly found in most eukaryotic cells and some prokaryotic cells, even in the nuclei of some cells, LDs can store neutral lipids that can be degraded by lipolysis mediated by LDs-associated lipases or lipophagy mediated by molecular chaperones that recognize proteins on the surface of LDs and transfer them to lysosomes for degradation (Murphy, 2012). Not merely limited to energy storage, LDs play an important role in many physiological and pathological processes within the body, such as preventing the accumulation of toxic substances in the endoplasmic reticulum

through regulation of the oxidative stress process, combining with mitochondria to participate in metabolic regulation, mediating cellular dysfunction and even modulating serious diseases caused by mutations in certain characteristic LD-related proteins (Welte and Gould, 2017).

Currently, the process of lipid droplet formation (**Figure 1**), which follows the process of transferring from the formation of the oil phase to the cytoplasm of the water phase, is not very clear, but it can be summarized into several conceptual steps: synthesis of neutral lipids and formation of lenses, grease bud, growth and maturation of LDs (Walther et al., 2017; Olzmann and Carvalho, 2019). Neutral lipid synthesis induced by Nem1p-Spo7p plays a very important role in maintaining the lipid balance of the ER membrane, particularly by preventing excessive accumulation of lipids (Karanasios et al., 2013). The FIT protein may affect germination and promote germination from ER-appropriate LDs. There is also evidence that nascent LDs form a lens-like structure in the ER membrane, and in the absence of FIT proteins, nascent LDs cannot germinate from the ER but grow and remain in the membrane (Choudhary et al., 2015). There may be multiple proteins involved in the budding process. For example, PERILIPIN (PLIN)-3 is considered to be the primary regulator of LD formation (Skinner et al., 2009). Studies have shown that PLD1 and ERK2 are essential for the formation of LDs and enhancing insulin stimulation (Andersson et al., 2006). Previous studies indicated that TIP47, with its lipoid properties that functions similar to recombinant liposomes, plays a role in the development of LD biogenesis because inhibition of TIP47 blocks the maturation of LDs and reduces the incorporation of trimethylphenyl glycerol (Bulankina et al., 2009). LDs grow and expand by acquiring specific proteins. Large LDs are formed by at least two general mechanisms: the process of LD growth or LD binding to form a single and larger LD. The LD expansion pathway is triggered by the activation of ARF1/COP-I proteins on the LD surface, forming nano LDs and removing primarily phospholipids from the LD surface, increasing the surface tension and enabling the modified LDs to interact with the endoplasmic reticulum. This process then allows the formation of ER-LD membrane bridges as well as the migration of TG synthase to the surface for LD expansion (Wilfling et al., 2014). LDs can also fuse to achieve amplification, including diffusion mediated by Ostwald maturation. The transcription factor MLX is one of the targets localized to LDs. By binding to LDs, MLX coordinates lipid storage by regulating the expression of metabolic genes (Mejhert et al., 2020).

Neutral lipids stored in LDs are primarily degraded by two pathways: lipophagy and lipolysis. LD degradation can selectively target LDs through autophagy-mediated lipolysis (macrophages) by hydrolyzing triglycerides (TGs) into free fatty acids (FFAs) as an energy source (Olzmann and Carvalho, 2019). The LD-related proteins Perilipin 2 (PLIN2) and Perilipin 3 (PLIN3) are autophagic substrates that undergo autophagy degradation before lipolysis (Kaushik and Cuervo, 2015). It was found that a decrease in autophagy promotes lipid accumulation and further inhibits autophagy, increasing lipid retention (Robichaud et al., 2021). Lysosomes do not directly fuse with LDs but with autophagosomes containing LDs (Singh, 2010). In addition, LDs can be decomposed into multiple fat vesicles by related lipases. Phosphatidylcholine prevents lipid droplet polymerization, and the rate-limiting enzyme in phosphatidylcholine synthesis is activated by binding to LDs.

Oxysterols, the hydroxylated metabolites of cholesterol, were first identified as intermediates in bile acid metabolism, but their pleiotropic roles in immunity and inflammation draw more attention (Spann and Glass, 2013; Cyster et al., 2014; Aguilar-Ballester et al., 2020). The oxysterols mainly include 25-hydroxycholesterol, 7α,25-dihydroxycholesterol, 27-hydroxycholesterol and 7α,27-dihydroxycholesterol (Willinger, 2019). The oxysterols can involve the regulation of the LDs in immune cells. The crosstalk between the 25-hydroxycholesterol and RORα can regulate the LDs homeostasis in macrophages (Tuong et al., 2016).

In general, LDs, organelles that store neutral lipids, play an important role in energy metabolism, preventing lipid toxicity and oxidative stress (Young et al., 2013; Bosma et al., 2014). LDs can also maintain endoplasmic reticulum homeostasis by storing excess lipids, regulating the metabolism and homeostasis of their lipids or protein cargo and playing a role in protein maturation, storage, and turnover. Additionally, LDs can also provide corresponding physiological sites to store fat-soluble vitamins, but for other vitamins, this process is not clear (Welte and Gould, 2017). More importantly, LDs act as bioactive lipids that regulate inflammation and immunity and are the hub that integrates metabolism and systemic immunity. Therefore, herein, we summarize the important role of LDs in multiple immune cells and analyze the role of the interaction between LDs and immunity in several related diseases.

Immunometabolism

The immune system provides defense, surveillance, and clearance functions for the body to prevent disease progression. While conducting these important functions, the immune system consumes considerable bioenergy. Rationally allocating energy materials for immune operation requires precise regulation of the metabolic pathways of immune cells. The study of immune metabolism can be traced back to a century ago when it was observed that immune cell metabolism is highly dependent on glucose (Levene and Meyer, 1912). The study of the immune metabolism of neutrophils and T cells began in the 1950s and 1960s. In the 1950s, studies found that neutrophils primarily activate respiratory bursts through glycolysis (Valentine and Beck, 1951; Sbarra and Karnovsky, 1959). In the 1960s and 1970s, glycolysis and glutamine breakdown were reported to be important in T cell activation, and mitotic lectins were observed to significantly stimulate the uptake of glucose and glutamine by lymphocytes (Parenti et al., 1966; Polgar et al., 1968; Roos and Loos, 1973; Hume et al., 1978). During the gradual exploration of metabolites, it was discovered that immune cells use metabolites for two important purposes: providing a substrate for ATP synthesis to maintain the activated state of the immune system and synthesizing macromolecular substances, such as DNA, RNA, protein, and cell membrane, for the immune system to function (Ganeshan and Chawla, 2014). Glucose is the primary substance involved in the energy

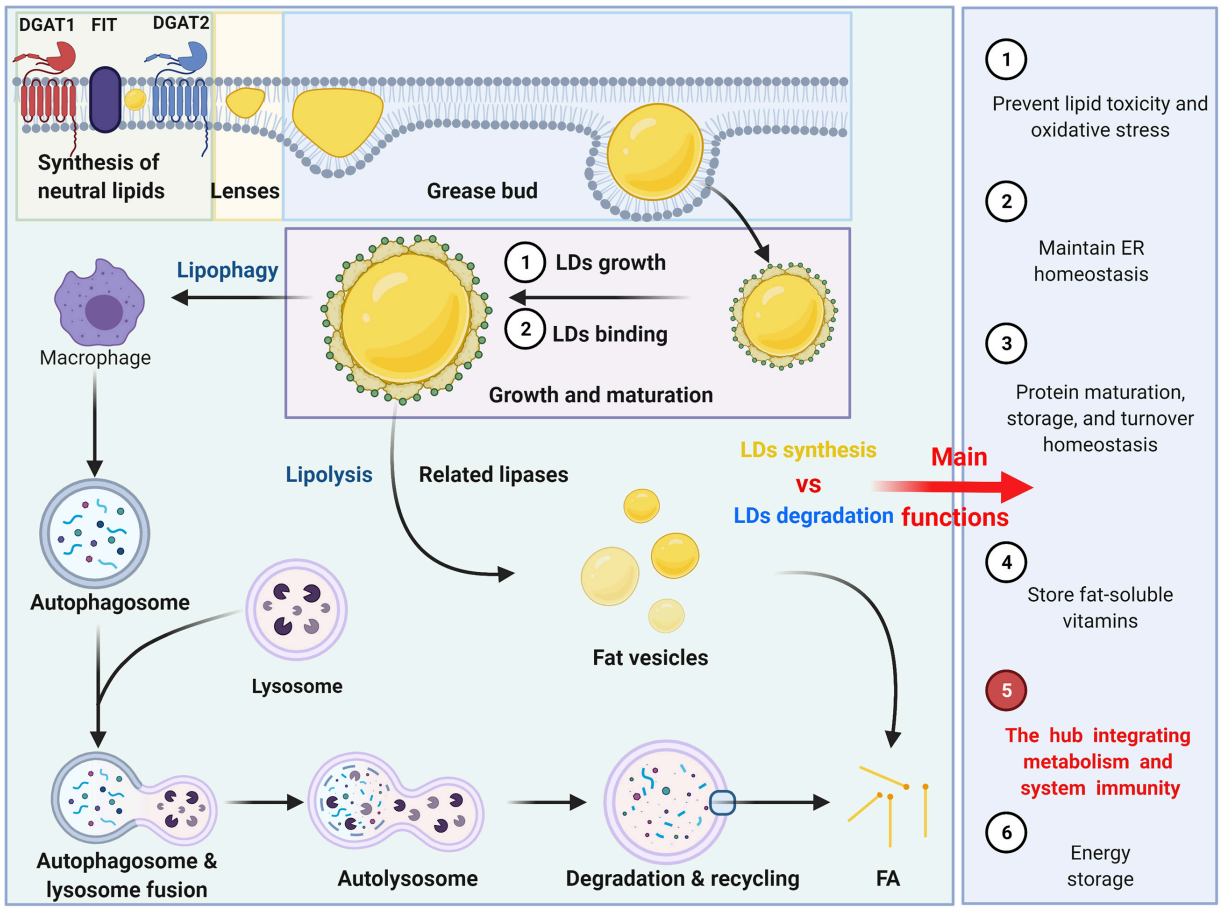

FIGURE 1 | The formation, degradation and main functions of LDs. The formation can be summarized into several conceptual steps: synthesis of neutral lipids and formation of lenses, grease bud, growth and maturation of LDs. The degradation of LDs can be divided into two ways: lipophagy and lipolysis.

metabolism of immune cells with lipids providing part of the energy. When immune requirements increase, T cells require extremely high levels of glycolysis. T cells express a large number of GLUT family members with the main participants including the kinase mammalian target of rapamycin complex 1 (mTORC1), transcription factor c-Myc, and hypoxia-inducible factor 1α (HIF-1α), thereby obtaining a large amount of energy for differentiation (Shi et al., 2011; Wang R. et al., 2011; Sinclair et al., 2013; Macintyre et al., 2014). The mechanism involves metabolic reprogramming (Sinclair et al., 2013). The difference is that the levels of glycolysis in Treg cells are relatively low, and fatty acid oxidation provides the primary fuel (Michalek et al., 2011). Further metabolic programming of memory T cells also depends on downregulation of glycolysis levels and conversion to lipid oxidation (Gubser et al., 2013). B cells primarily receive B cell receptor (BCR) stimulation, TLR4 stimulation, and IL-4 stimulation but not mTORC1 stimulation (Doughty et al., 2006; Dufort et al., 2007; Caro-Maldonado et al., 2014). Under these stimulatory conditions, B cell glycolysis or OXPHOS levels are upregulated, but the magnitude is not as high as that of T cells (Donnelly and Finlay, 2015). Aerobic glycolysis increases levels of the abovementioned immune cells to varying degrees, which not only promotes the biosynthesis of these cells but also has important significance for controlling cell differentiation and regulating effectors (Donnelly and Finlay, 2015). Lipids are not the primary functional source, but their participation in immune metabolism has attracted increasing attention. For example, the maintenance of immune cell quiescence, anti-inflammatory alternative macrophage activation, and CD8 memory formation is highly dependent on the β-oxidation of fatty acids and mitochondrial oxidative metabolism (Vats et al., 2006; Odegaard et al., 2007; Pearce et al., 2009). Despite rapid progress over the past century, our understanding of immune metabolism is still in its infancy. Limitations include incomplete identification of metabolic effectors in the immune cell bank, lack of understanding of inducers and sensors that activate immune cells, lack of consideration of the diversity of immune cell spectrum and tissue specificity, etc., (Ganeshan and Chawla, 2014). In the future, immune metabolism research will enhance or weaken the lethality of the immune system by changing the metabolic program of immune cells and provide us with treatment options for cancer and infections. This article elaborates on the role of LDs in immune cells. For innate immunity, we will focus on the role of LDs in neutrophils, mast

cells, macrophages and dendritic cells. For specific immunity, we will focus on the interaction between LDs and T cells.

LIPID DROPLETS AND IMMUNE CELLS

Lipid Droplets and Neutrophils

Neutrophils, derived from bone marrow stem cells, are part of the innate immune system. Known as polymorphonuclear granulocytes (PMNs) due to their lobulated nuclei, they are the most numerous white blood cells in the blood and are characterized by a short life, fast renewal, and high quantities. Neutrophils exert chemotactic, phagocytic, bactericidal, and other biological functions, which are primarily related to the many fine neutral particles evenly distributed in their cytoplasm. During acute inflammation, in addition to phagocytosis and killing bacteria, neutrophils can also perform chemotaxis and recruit other effector cells to the infected site through the synthesis and secretion of cytokines or the mechanism of mutual contact, regulating innate or adaptive immunity and further enhancing the killing effect of bacteria. In recent years, the role of neutrophils in addition to acute inflammation has attracted increasing attention. Interestingly, the cytokines released by neutrophils are primarily lipid mediators that include arachidonic acids. Therefore, the metabolic requirements of neutrophils have received increasing attention. However, compared to macrophages and T cells, there are few studies examining the metabolic characteristics of neutrophils, particularly lipid metabolism. Regardless of the type of immune cell, lipid metabolism primarily occurs in the mitochondria; however, it is worth noting that neutrophils have almost no mitochondria and perform glycolysis to produce energy in most cases.

In mammals, as a dynamic organelle that is attracting increasing attention, LDs are closely related to mitochondria. This association may be controlled by AMPK because AMPK activation causes LDs to spread across stable microtubules, which is thought to increase their interaction with mitochondria (Herms et al., 2015). Several recent studies have demonstrated that LDs are involved in various biological processes, particularly innate immunity. Therefore, it is not difficult to speculate that LDs play an extremely important role in the occurrence, development, and function of neutrophils.

A remarkable phenomenon observed during bone marrow differentiation into neutrophils is the accumulation of LDs (Inazawa et al., 2015). The formation of LDs or adipogenesis is an important part of neutrophil differentiation. LD formation is not only related to lipogenesis but is also closely related to fat degradation, all of which have been shown to affect the differentiation of neutrophils. Inhibition of galectin-12 in ATATA-induced human promyelocytic leukemia cell line NB4 inhibits lipid droplet formation *via* PPARγ and ultimately promotes the differentiation of neutrophils (Xue et al., 2016). Expression levels of PPARγ, p-CREB, C/EBPα, and C/EBPβ in galectin-12 knockdown cells are lower, all of which regulate lipid production (Xue et al., 2016). Notably, PPARγ is a key transcription factor that regulates adipogenesis, stimulates lipid production, and induces NB4 cell differentiation (Yasugi et al., 2006). In addition to adipogenesis, neutrophil differentiation is also closely related to fat degradation, which was recently confirmed in a study examining the mechanism of autophagy in neutrophil differentiation (Riffelmacher et al., 2017). The results of this experiment demonstrated that neutrophils deficient in Atg7 (an essential autophagy protein) exhibit increased glycolytic activity, impaired mitochondrial respiration, reduced ATP production, impaired differentiation, decreased lipolysis, and accumulation of LDs (Riffelmacher et al., 2017). Autophagy is closely related to centrifugal differentiation and metabolic conversion. During the process of normal neutrophil differentiation, autophagy can degrade LDs primarily by targeting lipids to lysosomal lipase to hydrolyze phagosomes. On the one hand, this prevents lipid accumulation and avoids lipotoxicity. On the other hand, the degraded LDs release free fatty acids to maintain the total amount of cytosolic fatty acids. Normal neutrophils undergo metabolic reprogramming from glycolysis to FFA-dependent oxidative phosphorylation during their development, and autophagy can decrease the levels of glycolysis in neutrophils and increase the levels of oxidative phosphorylation (Riffelmacher et al., 2017). In addition to autophagy, a dynamic hub of cellular lipid metabolism, LDs can isolate excess lipids and provide a source of FAs through LD-related neutral lipases, for example, ATGL. LD-related neutral lipase can respond to the metabolic state of cells, degrading TAG to produce abundant free FAs, which can be transferred to the mitochondria to produce a large amount of ATP to meet physiological needs. During the normal differentiation of neutrophils, lipid metabolism is closely related to cellular differentiation. However, the specific mechanism by which lipid metabolism, including lipidogenesis and lipolysis, affects neutrophil differentiation remains to be further studied.

As a key marker of leukocyte activation, the polymorphism of LDs during inflammation is well known, and their biogenesis is cell-type and stimulus dependent (Walther and Farese, 2012). When cells are activated, the number and size of LDs in neutrophils rapidly increase, mainly to prepare for the secretion of cytokines (Bozza and Bandeira-Melo, 2005). LDs can store the precursors of cytokines, primarily arachidonic acids, in neutrophils. Arachidonic acids primarily include prostaglandins, thromboxanes, and leukotrienes, which are derived from arachidonic acid (AA) and the related 20-carbon polyunsaturated fatty acid (Bozza et al., 2011). Free AA can be obtained by enzymatic decomposition of membrane phospholipids or can be released from AA-rich triglycerides stored in LDs in some cells. LDs modulate the immune response in neutrophils, as impaired LD lipolysis is accompanied by reduced arachidonic acid release (Schlager et al., 2015). Since arachidonic acid is a non-storable mediator that is rapidly formed and released after cellular stimulation, the release intensity of bioactive lipid mediators in neutrophils depends on the amount of LDs. Prostaglandin E2 (PGE2), the most important bioactive mediator secreted by neutrophils, accelerates blood flow and produces oedema and pain, and its synthesis is primarily related to AA, which is related to LDs (Kawahara et al., 2015). As a key signaling molecule in lipid metabolism in neutrophils, AA acts as a second messenger

to transmit signals in cells and can also be converted into prostaglandin E2 by a variety of enzymes. During prostaglandin E2 synthesis, three main enzymes are involved: phospholipase A2 (PLA2), cyclooxygenase (COX-1 and COX-2), and terminal prostaglandin synthase. Notably, phospholipids can be cleaved by phosphorylated cytoplasmic PLA2 to produce free fatty acids, including AA, which in turn forms prostaglandins G2 (PGG2), PGH2, and PGE2, catalyzed by cyclooxygenase (COX-1 and COX-2) and terminal prostaglandin synthase (Gupta and Dubois, 2001; Bapna and Chauhan, 2015). Cr-LAAO is an L-amino acid oxidase isolated from venom that stimulates neutrophil activation and arachidonic acid production (Pontes et al., 2014). During the activation of neutrophils induced by Cr-LAAO, the microarray data also demonstrated that the expression of lipid-related genes was upregulated not only due to signal transduction and metabolism genes, such as DGAT1, DGAT2, cPLA2-α, and COX-1. COX-2 and prostaglandin E synthase, as well as others, also include genes related to lipid droplet formation, including PLIN2, PLIN3, DGAT1, and DGAT2 (Paloschi et al., 2020). Perilipin 3 (PLIN3) plays a vital role in the formation of LDs in neutrophils and in the release of PGE2 because when PLIN3 is downregulated by siRNA treatment, LDs in HL-60-derived neutrophils disappear, the secretion of PGE2 levels decreases by 65%, and the enzymes involved in the synthesis of PGE2 are also inhibited (Nose et al., 2013).

Overall, the formation of LDs is not only related to lipogenesis but is also closely related to fat degradation, all of which not only affects neutrophil differentiation but also participates in the immune response as precursors of stored cytokines (primarily arachidonic acid) in neutrophils (**Figure 2**).

Lipid Droplets and Mast Cell

Mast cells, derived from myeloid stem cell precursors, play a key role in the initiation, maintenance, and resolution of the inflammatory response and are primarily involved in hypersensitivity and antiparasitic infection *in vivo*. After activation, mast cells release a variety of cytokines and chemokines, including histamine, serotonin, and arachidonic acid, playing a wide range of pathophysiological roles in a variety of diseases, including atherosclerosis, rheumatoid arthritis, and asthma (Abraham and St John, 2010; Galli and Tsai, 2012). Mast cells can be divided into two types: mucosal cells, primarily distributed in the submucosa with proliferation dependent on T cells, and connective tissue mast cells, primarily distributed in the connective tissues around subcutaneous small blood vessels and independent of T cells. Mast cells are phenotypically and functionally heterogeneous, depending on the microenvironment in which they mature. There are two main ways mast cell undergo activation: PPR-mediated and IgE-mediated. The PRR on the surface of mast cells recognizes the PAMPs of pathogenic microorganisms and then initiates the activation signal. In addition, mast cells express high-affinity IgE Fc receptors (FcεRIs) on their surface that can be activated by corresponding ligands. Mast cells contain two specialized proinflammatory organelles: secretory granules containing histamine, serotonin, and matrix active protease and cytoplasmic liposomes, which are the sites of arachidonic acid metabolism, precursor storage, and synthesis in mast cells (Galli and Tsai, 2010; Melo et al., 2011). When mast cell FcεRIs cross-link with each other through the phosphorylation of ITAM at the C-end of the γ chain mast cells are activated to perform physiological functions. On the one hand, the phosphorylation of ITAM activates SYK and Fyn tyrosine kinase and activates the PI-PLCγ signal chain to phosphorylate the light chain of myosin in the cytoplasm, leading to degranulation and release of biologically active mediators. Activated mast cells cannot exert a direct killing effect on pathogenic microorganisms entering the body, but they can quickly degranulate, release inflammatory factors in the granules into surrounding tissues, recruit immune effector cells, such as neutrophils and basophils, to reach the site of infection, and initiate a local inflammatory response. On the other hand, phosphorylation of ITAM activates the mitogen promoter protein (MAK) kinase signaling pathway, decomposing membrane phospholipid choline (PC) to produce arachidonic acid, which then passes through the cyclooxygenase and lipoxygenase pathways to synthesize prostaglandin D2 and leukotrienes and decompose alkylated phospholipids to generate LYSO-PAF, which is converted into platelet-activating factor (PAF) through the action of acetyltransferase. Prostaglandin D2, leukotriene, and platelet-activating factors are lipid mediators, collectively known as arachidonic acids, which induce differential biological effects. The activation of mast cells and PGD2 secreted by mast cells can affect the activation of leptin-induced eosinophils in humans because studies have shown that leptin-induced eosinophils in sensitized BALB/c mice depends on PGD 2 derived from mast cells (Amorim et al., 2020). The accumulation of liposomes in mast cells was associated with increased levels of leukotriene synthase (LTC4S and 5-LO) because studies have shown that insulin-containing adipogenesis stimulators can inhibit the degranulation potential of mast cells and upregulate the biogenesis of liposomes and the secretion of eicosanoids (Greineisen et al., 2012). As TGs in cytoplasmic LDs are the primary storage sites of AA and because ATGL can hydrolyze the AA-containing TG that exists in the LD of human mast cells, silencing of ATGL expression can lead to a significant accumulation of neutral lipids in the cytoplasm of mast cells and consequently reduce the production of eicosanoids by these cells. In addition to storing lipids, the accumulation of LDs in mast cells can change the dynamics of calcium signals, accelerating the spread of calcium ions, which affects the intensity and characteristics of the calcium response after antigen exposure stimulation (Greineisen et al., 2014). There is a close physical and functional interaction between liposomes and mitochondria (Klecker et al., 2017; Valm et al., 2017; Gemmink et al., 2018). The lipid droplet-associated proteins Perilipin 5 and SNAP23 are reported to play a crucial role in the physical and metabolic connections between liposomes and mitochondria (Wang H. et al., 2011). Chen et al. (2016) used soft X-ray tomography (SXT) combined with fluorescence microscopy to study the detailed structure of organelles during mast cell activation and observed granular fission, granular-cell membrane fusion, and small vesicles sprouting from the granules. Mast cell degranulation involves multiple membrane events, including particle fusion and fission and particle and membrane fusion, and these processes require intracellular calcium signaling and

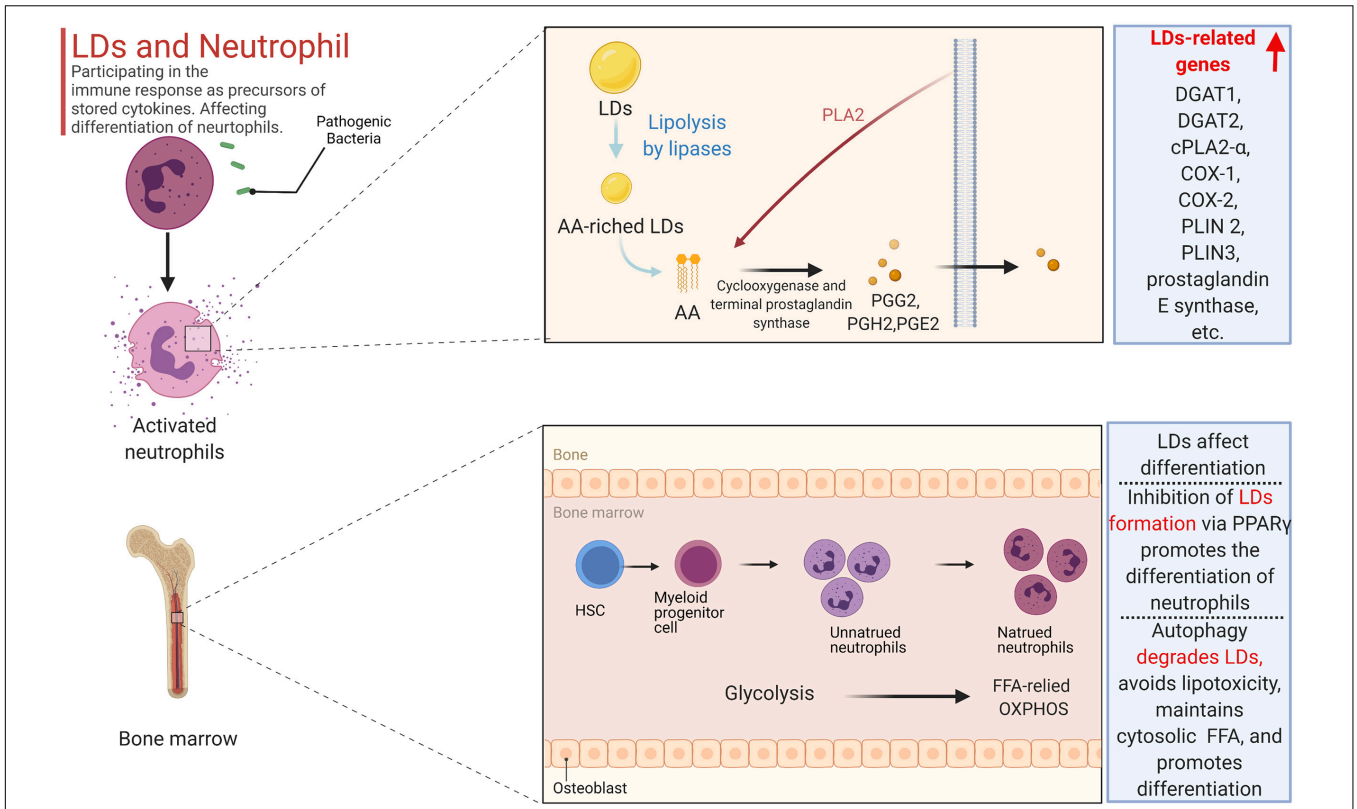

FIGURE 2 | The formation of LDs is not only related to lipogenesis but also closely related to fat degradation, all of which not only affects neutrophil differentiation but also participates in the immune response as precursors of stored cytokines (primarily arachidonic acid) in the neutrophil. Free AA can be obtained by enzymatic decomposition of membrane phospholipids or can be released from AA-rich triglycerides stored in LD in some cells. Phospholipids can be cleaved by phosphorylated cytoplasmic PLA 2 to produce free fatty acids including AA, which in turn forms prostaglandins G2 (PGG 2), PGH 2, and PGE2, catalyzed by cyclooxygenase (COX-1 and COX-2) and terminal prostaglandin synthase. In the activation of neutrophils induced by Cr-LAAO, the microarray data also proved that the expression of lipid-related genes was up-regulated. Inhibition of galectin-12 in ATATA-induced human promyelocytic leukemia cell NB4 inhibits lipid droplet formation *via* PPARγ, and ultimately promotes the differentiation of neutrophils. Autophagy can degrade LD, not only preventing lipid accumulation and avoiding lipotoxicity, but also releasing free fatty acids to maintain the total amount of cytosolic fatty acids.

mitochondrial function. LD biogenesis by mast cells in the body is not only closely related to the ER but is also affected by hormones in the body, especially insulin. In RBL2H3, a reliable model of mast cells, exposed to insulin for a long time, liposomes excessively accumulate in the cell, and various markers of UPR (IRE1α, phosphorylated PERK, and ATF6) are upregulated, leading to the ER response. At the same time, the expression of proteins related to autophagy, such as ATG3, ATG12, ATG7, Beclin, and LC3A, has also been observed to increase accordingly (Greineisen et al., 2015).

In general, the role of lipid droplets in mast cells is primarily to act as the site of metabolism, storage, and synthesis of lipid inflammatory mediators and to change the dynamics of calcium signaling to affect the degranulation of mast cells and the secretion of inflammatory mediators (**Figure 3**).

Lipid Droplets and Macrophages

In general, having a very heterogeneous cell population that exhibits unique phenotypes and functions in the complex microenvironment of the body, macrophages are the hub that connects innate and adaptive immunity in the body. In contrast to monocytes in the blood after passing through blood vessels, macrophages are derived from precursor cells in the bone marrow. They exist throughout the body and participate in the inflammatory response as the blood circulates throughout the body. Although the morphology and function of macrophages are heterogeneous, which is determined by the macrophage microenvironment composed of a variety of cytokines, cells, pH, and oxygen content, their main function is to participate in the phagocytosis of various substances in the body, such as bacteria, viruses, fungi, parasites, cell debris, and tumor cells, participating in innate immunity and, at the same time, as antigen-presenting cells that absorb, process and present antigens to T cells, participating in adaptive immunity. Depending on their phenotype and function, macrophages can be roughly divided into two categories: M1 and M2. M1 type macrophages induced by tumor necrosis factor (TNF)-α, interferon (IFN)-γ) or invading pathogens secrete chemotactic and proinflammatory cytokines (IL-1β, TNF-α, and IL-12), present antigens, participate in Th1-mediated positive immune response and perform immune surveillance; M2-type macrophages have only weak antigen-presenting ability and play

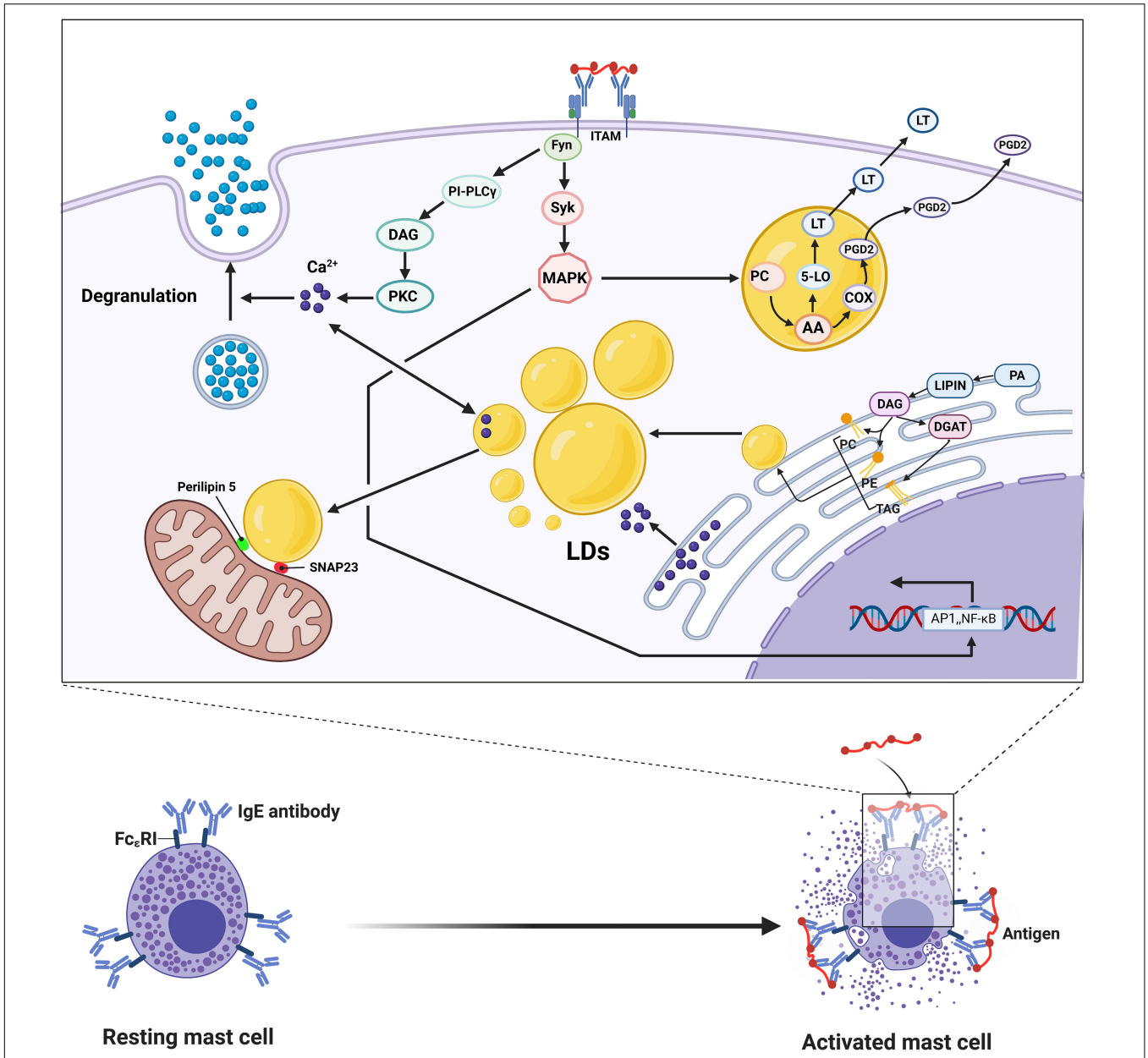

FIGURE 3 | IgE cross-linking mediated activation of mast cells leads to degranulation and release of large amounts of bioactive lipids, including prostaglandin D2 and leukotriene (LT). After FcεRI is cross-linked with each other, the phosphorylation of ITAM activates Fyn and Syk, which in turn mediates activation of PKC through the PI-PLCγ signaling chain, resulting in increased intracellular calcium concentration and phosphorylation of the intracytoplasmic myosin light chain, leading to degranulation and release of bioactive mediators. Phosphorylation of ITAM activates the mitogen-activated protein (MAK) kinase signaling pathway, causing the breakdown of phospholipid choline (PC) in the presence of phospholipase A2 to produce arachidonic acid (AA), which is then combined into PGD2 and LT, respectively, *via* the cyclocythase and lipoxygenase pathways. Activation of MAPK also increases the transcription of several nuclear transcription factors, such as NF-κB and AP1. After FcεRI is cross-linked, LDs are not only the source of calcium ions in mast cells but also the sinks of calcium ions, acting as the source and absorber of calcium ions, thereby affecting the corresponding signal pathways. The LDs-associated proteins Perilipin 5 and SNAP23 play crucial roles in the physical and metabolic connections between LDs and mitochondria. The synthesis of LDs is mainly carried out in the endoplasmic reticulum, accompanied by the synthesis of TAG, PC, and PE with FA as the substrate under the action of various enzymes.

an important role in Th2-dependent immune regulation by secreting inhibitory cytokines, such as IL-10 and TGF-B, to downregulate the immune response (Mehla and Singh, 2019).

In recent years, multiple studies have shown that LDs and macrophages are closely related to diverse functions in different microenvironments, such as atherosclerosis, tumor microenvironments, and infection (parasites, viruses, bacteria, and fungi).

Macrophages can reduce excessive lipid accumulation in the body by increasing the lipolysis of intracellular LDs,

avoiding damage to cells from lipid toxicity and alleviating the further development of atherosclerosis. The primary mechanisms include the miR-328-5p reduction-mediated histone deacetylase 3/ATP-binding cassette transporter A1 pathway (Huang et al., 2021), lipid droplet ubiquitination mediated by AUP1 (Robichaud et al., 2021), TAG synthesis guided by GPAT3 and GPAT4 (Quiroga et al., 2021), hydrolysis of cholesterol ester catalyzed by neutral cholesterol ester hydrolase 1 (Matsuoka et al., 2020), Foxc2-induced Angptl2-mediated lipid accumulation (Yang L. et al., 2020), inhibition of mitochondrial respiration by iNOS-derived nitric oxide (Rosas-Ballina et al., 2020), SphK2-affected lipid droplet decomposition mediated by autophagosomes and lysosome (Ishimaru et al., 2019), BECN1 and ATG14 mediated autophagy (Singh et al., 2009; Ouimet et al., 2011; Hadadi-Bechor et al., 2019), lipid accumulation marked by the perilipin family of PLIN1-PLIN5 (Sztalryd and Brasaemle, 2017; Bosch et al., 2020), LD biogenesis mediated by PPAR signaling pathway (Yang T. et al., 2020), acute iron deprivation (Pereira et al., 2019), neutral lipase-mediated hydrolysis of neutral fat in LDs (van Dierendonck et al., 2020), and fat phagocytosis mediated by Hmgb1, Hmgb2, Hspa5 and Scarb2 (Robichaud et al., 2021; **Figure 4**). HILPDA is a physiological inhibitor of ATGL-mediated lipolysis in macrophages that binds to the intracellular triglyceride hydrolase ATGL and inhibits ATGL-mediated triglyceride hydrolysis (van Dierendonck et al., 2020). Retinoic acid receptor-related orphan receptor α induces high expression of NCEH1 in macrophages, promoting the hydrolysis of cholesterol ester in LDs and alleviating lipid accumulation (Matsuoka et al., 2020). In addition, MLX transcription factors and LDs can bind to each other on the surface of LDs, which can alter the storage level of LDs in macrophages to meet the metabolic needs of cells in the current microenvironment (Mejhert et al., 2020). When there are too many LDs, it can weaken the metabolic pathway that uses glucose as the substrate, and when LDs are too low, MLX can regulate metabolic genes such as AQP3 and increase the storage of LD (Mejhert et al., 2020). Macrophage autophagy can enhance lipid decomposition by lipohydrolase and reduce LD levels, alleviating some metabolic diseases related to elevated lipid levels, such as atherosclerosis. Interestingly, recent studies have shown that macrophage autophagy appears to be heterogeneous, and different lipid substrates or different inflammatory activation modes may lead to differences in the levels of macrophages processing LDs (Hadadi-Bechor et al., 2019).

Compared to macrophages in normal tissues, tumor-associated macrophages (TAMs) located in the tumor microenvironment various distinct characteristics and functions, including inhibiting antitumor immunity and promoting tumor cell proliferation, transformation, escape, and metastasis (Conway et al., 2016). Cancer cells tend to release fatty acids into the surrounding environment to form a fatty acid-rich tumor microenvironment. Different types of TAMs located in this environment have different metabolic pathways. Glycolysis of proinflammatory macrophages increases; however, anti-inflammatory macrophages, with increased FAO mediated by PPARγ, PGC-1β, and STAT6, exhibit the characteristics of M2 phenotype macrophages and have a strong ability to inhibit antitumor immunity and promote tumorigenesis and development (Mehla and Singh, 2019). LDs play a very important role in the formation of the anti-inflammatory phenotype of TAMs, regulating lipid synthesis and metabolism, and the free FAs produced by metabolism can be used as a substrate for mitochondrial productivity, thereby regulating oxidative phosphorylation of mitochondria (Boeszoermenyi et al., 2015). Of note, the phenotype of tumor-associated macrophages is not static. M1 and M2 subtypes can be interconverted. Considering the proinflammatory characteristics of M1 macrophages and the tumor-promoting characteristics of M2 macrophages, we hope to improve the tumor microenvironment and promote the transformation of tumor-related macrophages from the M2 type, which suppresses antitumor immunity, to the M1 type, which promotes the inflammatory response. Studies have shown this possibility. Bose et al. (2019) demonstrated through RAW264.7 cells that TGF-β induces the formation of LDs in tumor-associated macrophages, and the transformation of M2 macrophages to M1 macrophages was observed when TGF-β-induced LDs were inhibited. Further experiments showed that this may be related to the MEK1/2 axis (Bose et al., 2019). It is not difficult to see that the M2 type of TAM FAO has a strong demand, so targeting the PPARγ, PGC-1β, and STAT6 signaling pathways to reduce their mediated FAO is likely to be an effective strategy for promoting the transformation of M2 macrophages to M1 macrophages (Mehla and Singh, 2019).

In addition, macrophages are involved in the body's immunity against infection. LDs, the accumulation of which is a sign of infection, also play a very important role in this process. However, there is still much controversy over whether LDs promote the body's resistance to infection or support the body's susceptibility to infection. Previous views have held that LDs support infection primarily by providing fatty acids as substrates of FAO to produce large amounts of ATP for bacteria and providing pathogenic microorganisms with lipids to synthesize their membrane structure to ensure the normal structure and function of their membrane structure, promoting infection persistence and further development (Allen and Martinez, 2020). Studies have shown that *Mycobacterium tuberculosis* can persist in the body, which is closely related to the activation state and metabolic pathways of its host cells (Genoula et al., 2020). In particular, the results of this study indicated that the continuous accumulation of LDs mediated by STAT6 in foam macrophages (a type of macrophage rich in LDs is a sign of continuous *Mycobacterium tuberculosis* infection) is beneficial to tuberculosis. The long-term existence of *Mycobacterium tuberculosis* in the body promotes the further development of infection (Jaisinghani et al., 2018; Guerrini and Gennaro, 2019; Genoula et al., 2020). The accumulation of LDs is related to host-pathogen interactions. NR1D2-mediated PNPLA2/ATGL inhibition disrupts the lipid metabolism of macrophages in the human body, leading to lipid accumulation and weakening the effect of autophagy on *Burkholderia pseudomallei* immunity to infection, leading to the continued development of infection (Tang et al., 2021). In *Mycobacterium tuberculosis*, the interaction between LDs and pathogen-containing phagosomes is controlled by mycobacterial cell wall components (LAM and PIM) and

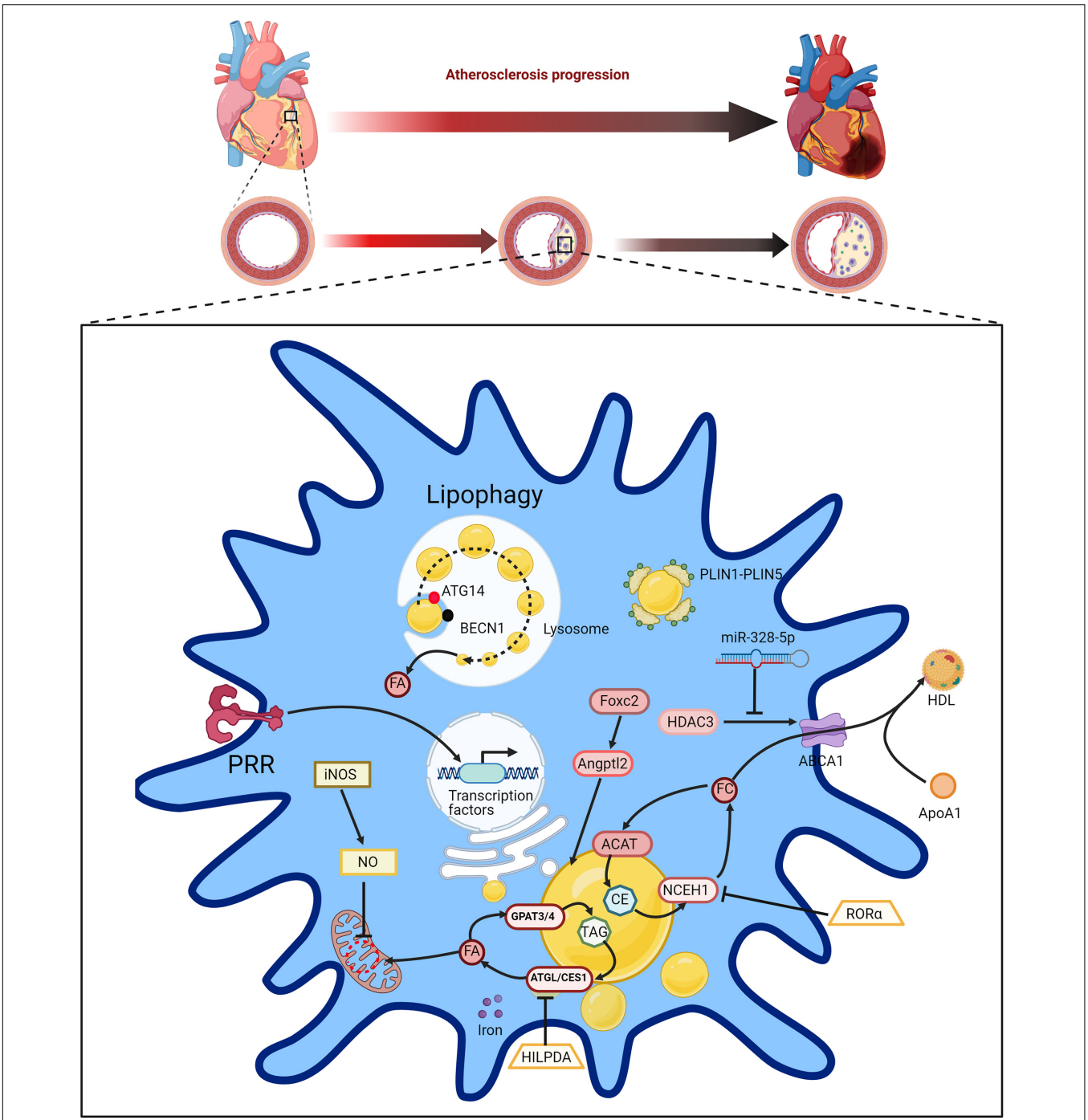

FIGURE 4 | Atherosclerosis is accompanied by the accumulation of LDs in macrophages. Macrophages can reduce excessive lipid accumulation in the body by increasing the lipopolysis of intracellular LDs and avoiding the damage of cells from lipid toxicity, thus alleviating the further development of atherosclerosis. Intracellular free cholesterol (FC) can be transferred extracellular by ABCA1 to combine with extracellular apolipoprotein A1 (ApoA1) to form high-density lipoprotein (HDL). The activity of ABCA1 is mediated by miR-328-5p reduction regulated histone deacetylase 3 (HDAC3). TAGs can be synthesized by FA under the action of GPAT3 and GPAT4, and TAGs break down to produce free fatty acids under the action of ATGL and CES1. The hydrolysis of cholesterol ester (CE) is catalyzed by neutral cholesterol ester hydrolase 1 (NCEH1) to produce free cholesterol (FE), and the synthesis of CE is catalyzed by acyl-coenzyme A-cholesterol acyltransferase (ACAT). Foxc2-induced Angptl2-mediated lipid accumulation is also associated with atherosclerosis. Nitric oxide (NO) derived from iNOS can inhibit mitochondrial respiration. The lipophagy can be mediated by BECN1 and ATG14 in macrophages, decomposing LDs to produce FA. The perilipin family of PLIN1-PLIN5 is the mark in LDs accumulation in macrophage. Hypoxia-inducible lipid droplet-associated protein (HILPDA) is a physiological inhibitor of ATGL-mediated lipopolysis in macrophages, binding to the intracellular triglyceride hydrolase ATGL and inhibiting ATGL-mediated triglyceride hydrolysis. Retinoic acid receptor-related orphan receptor α(RORα) can induce the high expression of NCEH1 in macrophages, thereby promoting the hydrolysis of cholesterol ester in LDs and alleviating lipid accumulation.

Rab7 (Roque et al., 2020). However, the results of a recent study indicated that to deal with infections, LDs in mammals assemble antibiotics and immune proteins to form a complex that fights pathogens and destroys them (Bosch et al., 2020). This mechanism is not unique to immune cells such as macrophages but also occurs in other cells in the body (Bosch et al., 2020). Through gene interaction analysis, the experimental results showed that immune proteins that can fight pathogens primarily gather around LD surface protein 2 (PLIN2) (Bosch et al., 2020). Therefore, the anti-infection effect of LDs on the body and the specific mechanism needs to be further studied and elucidated.

Overall, the interaction between LDs and macrophages is heterogeneous, which means that they exhibit different functions in different microenvironments, such as atherosclerosis, tumor microenvironments, and infection. Macrophages can reduce excessive lipid accumulation in the body *via* multiple mechanisms to alleviate the further development of atherosclerosis. LDs play a very important role in the formation of the TAM anti-inflammatory phenotype (M2) and are a potentially effective target for promoting the transformation of tumor-related macrophages from M2 to M1. Macrophages are also involved in the body's immunity against infection, but whether LDs promote the body's resistance to infection or support infection remains controversial.

Lipid Droplets and Dendritic Cells

Dendritic cells (DCs) originate from pluripotent hematopoietic stem cells in the bone marrow and are widely distributed throughout the body, but their concentrations are quite low. Representing the link between adaptive and innate immunity, the most important function of DCs is to absorb, process, and present antigens, stimulating the body to produce an immune response and regulating the body's immune response. According to phenotype and function, DCs can be divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Conventional DCs primarily release IL-12 after being stimulated with bacterial components and mainly express TLR2, TLR3, TLR4, and TLR5, and their main function is to induce a specific immune response against invading antigens and maintain self-tolerance (Chistiakov et al., 2019). Loss of rapamycin complex 2 (TORC2) leads to the proinflammatory phenotype of cDCs and increases the stimulation of T cells (Watson et al., 2019). The primary function of pDCs is to produce large amounts of type I interferon against microorganisms, especially viral infections, and to stimulate corresponding T cell responses. Under the stimulation of unmethylated CPG motifs derived from viruses and bacteria, pDC pits produce high levels of type I interferons, especially IFN-α, through the TLR7/9 signaling pathway, which directly interferes with viral replication and activates mononuclear macrophages to kill pathogenic microorganisms (Hedrick, 2015; Chistiakov et al., 2015b). DCs can absorb antigens through receptor-mediated endocytosis, macrophage cytosis, and phagocytosis, as well as process antigens in cells to clarify the pathogenic microorganisms, products, and harmful antigenic substances that invade the body. Through antigen presentation, DCs directly or indirectly regulate T and B cells, which primarily depends on costimulatory molecules, adhesion molecules, and antigen peptide/MHC molecular complexes on the surface of DCs, as well as secreted cytokines.

In immune cells, LDs are considered structural markers of inflammation (Bozza et al., 2007). Pathogen invasion is often accompanied by the accumulation of LDs (**Figure 5**). When host cells recognize pathogens through PRR, especially antigens through the TLR family, LDs accumulate in immune cells. This accumulation of LDs not only reflects the excess energy in DCs but also suggests to a certain extent that there is metabolic reprogramming in DCs. Resting DCs primarily use free fatty acids as substrates for oxidative phosphorylation through fatty acid oxidation to generate energy to meet physiological needs. Glucose can also be used as a substrate to produce energy in resting DCs, mainly because glucose can be converted to pyruvate by glycolysis and then enter the mitochondrial TCA cycle (Krawczyk et al., 2010). However, when DCs are activated, their metabolic mode markedly changes, becoming dominated by an aerobic glycolysis capacity similar to that of tumors, which is called the Warburg effect; that is, pyruvate produced by glycolysis no longer enters the TCA and is instead converted into lactic acid under harsh environments, such as hypoxia (Kelly and O'Neill, 2015). In cDCs, mTORC2-deficient DCs exhibited enhanced glycolysis and a proinflammatory phenotype and enhanced T cell activation (Watson et al., 2019). DC activation mediated by TLRs eventually leads to the accumulation of LDs, primarily due to metabolic reprogramming. This metabolic change process can be divided into the first stage of increased glycolysis to support the *de novo* synthesis of lipids and the second stage of glycolysis to produce lactic acid and ATP (Everts et al., 2014). Accumulation of citrate in DCs and the biosynthesis of prostaglandin are landmark events that occur during the metabolic reprogramming of DCs activated by TLR (O'Neill, 2014).

Lipid Droplets in normal immune cells seem to be related to immune enhancement (Bosch et al., 2020). For example, liposomes promote cross-presentation through phagocytosed antigens by MHC class I in DCs (Bougneres et al., 2009). However, in tumors, the increased intracellular lipid content of tumor–infiltrating DCs is observed more often (Yin et al., 2020). In addition, tolerable lipid DCs have been found in malignant microenvironments (Herber et al., 2010). Thus, LDs in DCs cannot be generally considered proinflammatory or anti-inflammatory because the function of LDs is primarily determined by lipid-associated proteins and internal lipids, which are closely related to the local microenvironment. The tumor microenvironment (TME) is characterized by hypoxia, nutritional deficiencies, reactive oxygen or nitrogen species, and elevated levels of adenosine, lactic acid, and immunosuppressive factors such as IL-10 and PD-L1 (Veglia and Gabrilovich, 2017). The cross-antigen presentation of DCs in the tumor microenvironment is weakened (Herber et al., 2010), but the specific mechanism whereby this occurs remains unclear. However, multiple factors are related to this phenomenon. The harsh local microenvironment impairs endoplasmic reticulum folding in cancer cells and tumor-infiltrating DCs, leading to ER stress, which is closely related to the accumulation of LDs in DCs. In this case, ER stress may be an intermediate pathological process induced by LDs in cancer immune cells.

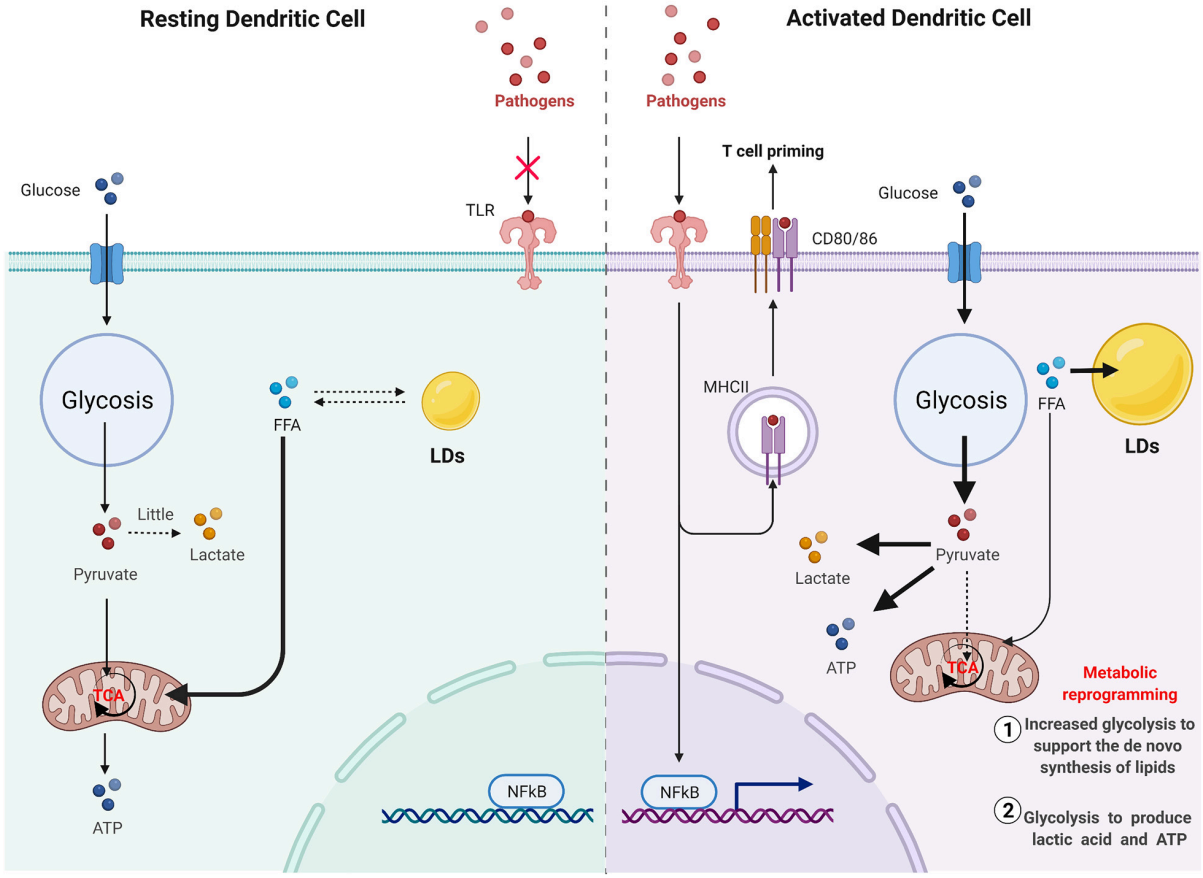

FIGURE 5 | Pathogen invasion is often accompanied by the accumulation of LD. Resting DC mainly uses free fatty acids as substrates for oxidative phosphorylation through fatty acid oxidation to generate energy to meet physiological needs. Glucose can also be used as a substrate to produce energy in resting DC, mainly because glucose can be converted to pyruvate by glycolysis, and then enter the mitochondrial TCA cycle. However, when DC cells are activated, pyruvate produced by glycolysis will no longer enter TCA and will be converted to lactic acid capacity under a harsh environment such as hypoxia. DC activation mediated by TLR will eventually lead to the accumulation of LD, mainly due to metabolic reprogramming. This metabolic change process can be divided into the first stage of increased glycolysis to support the *de novo* synthesis of lipids and the second stage of glycolysis to produce lactic acid and ATP.

Silencing of the endoplasmic reticulum stress connector XBP-1 in cancer-associated dendritic cells results in a reduction in the number and size of LDs in each cell, improved immune capacity, antitumor effects, and ultimately prolonged survival (Cubillos-Ruiz et al., 2015). Tumor cells use a variety of signaling molecules to communicate with DCs (DeVito et al., 2019). Exosomes are an important part of the tumor microenvironment and can play a role in signal transduction between tumor cells and non-tumor cells. Recent studies have shown that, compared to normal exosomes, the FA content of exosomes in the tumor microenvironment is increased, which can lead to LD accumulation in DCs and upregulation of mitochondrial FAO through activation of PPARα, resulting in the metabolic transition to mitochondrial oxidative phosphorylation and driving immune dysfunction in DCs (Yin et al., 2020). In addition to exosomes, tumor cells can also utilize paracrine Wnt5a/β-catenin signaling to activate PPARγ in DCs, enhance FAO and lead to DC dysfunction (Ramakrishnan et al., 2014). Mesothelioma cells exposed to acidosis in the tumor microenvironment promote the secretion of TGF-β2, and TGF-β2-dependent LDs accumulate in metabolic reprogramming DCs (Trempolec et al., 2020). Saponin-based adjuvants (SBAs) can induce intracellular liposomes in a subset of CD11b+ DCs, increasing the antigen presentation of DC cells (den Brok et al., 2016). Oxidized lipids prevent cross-presentation by reducing the expression of MHC class I peptide complexes on the cell surface, and the accumulation of non-oxidized lipids does not affect cross-presentation (Ramakrishnan et al., 2014). By combining lipidomics and molecular dynamics simulations, Veglia et al. (2017) found that liposomes with DC accumulation in tumor-carrying hosts contain electrophilic oxidized trapping (ox-TR) lipids that covalently bind to heat shock protein 70, and this interaction prevents pMHC transport from late endosomal/lysosomal transport to the cell surface. Oxidized lipids only affect the surface level of pMHC-1 with exogenous but not endogenous peptides (Ramakrishnan et al., 2014).

In addition to tumors, the relationship between LDs and DCs also plays a key role in the occurrence and progression of diseases

in other diseases. Hypoxia in the presence of obesity induces the activation of the HIF1α transcription factor, which can induce lipid accumulation in DC cells, inhibiting CDC activation, promoting an anti-inflammatory phenotype, and leading to the formation of atherosclerotic plaques and the enhancement of adipose tissue inflammation (Wood et al., 2020). Mouse bone marrow-derived DCs can be activated by the flagellum of Leishmania protozoa and undergo metabolic reprogramming to induce lipid accumulation (Lecoeur et al., 2013). Allithiamine has been identified as a coactivator of the PDH complex (PDC), which promotes the conversion of cytosolic pyruvate to mitochondrial acetyl-CoA, inhibits the decomposition of pyruvate into lactic acid, and regulates metabolic pathways during the activation of dendritic cells. It inhibits the production and maturation of proinflammatory cytokines in dendritic cells induced by lipopolysaccharide (LPS), exerting a therapeutic effect in sepsis (Choi et al., 2020).

Overall, in the TME, LDs in DCs cannot be generally considered proinflammatory or anti-inflammatory because the function of LDs is primarily determined by lipid-associated proteins and internal lipids, which are closely related to the local microenvironment. In addition, the cross-antigen presentation of DCs in the tumor microenvironment is weakened. The relationship between LDs and DCs also plays a key role in the occurrence and progression other diseases, such as obesity, infection, and sepsis.

Lipid Droplets and T Cells

The cells of the innate and adaptive immune systems, including T cells, have been shown to rely on different aspects of lipid metabolism to develop and function (Howie et al., 2017b). The microenvironment, including cytokines and costimulatory signal molecules, guides T cells to develop along different functional pathways. To a large extent, this is due to the distinct responses of T cell subpopulations to different metabolic stresses. LDs are derived from the endoplasmic reticulum, and DGAT1 and 2 esterify free acylated fatty acids to diacylglycerols to form more inert triacylglycerols, which are stored in the LD core. DGAT1 is responsible for the esterification of exogenous fatty acids into TG, while DGAT2 is considered to have advantages in the esterification of endogenous synthetic FAs (Bhatt-Wessel et al., 2018).

Th1, Th2, and Th17 effector T cells primarily rely on glucose uptake and glycolysis to function and maintain their stability, while regulatory T cells, a class of immunosuppressive Foxp3+ T cells, do not rely on glycolysis, and their mitochondrial oxidative phosphorylation and FAO levels are high (Huynh et al., 2015). Regulatory T cells (Tregs) are a subset of T cells that inhibit the activation and proliferation of other immune cells and play an important role in the suppression of rejection. Acetyl-coenzyme A carboxylase 1 (ACC1) is key for fatty acid generation in T cells. In addition, regardless of whether it is for mice or humans, inhibiting ACC1 not only inhibits fatty acid synthesis but also interferes with glucose metabolism through glycolysis and TCA, which can inhibit proinflammatory Th17 cells and CD8+ T cell induction and proliferation and promote the production of Foxp3 (+) Treg cells with immunosuppressive function, suggesting that endogenous fatty acid synthesis is needed for Th17 cells to develop (Berod et al., 2014). In the absence of exogenous long-chain free fatty acids, the formation of Foxp3+ Treg cells induced by TGFβ is inhibited (Michalek et al., 2011). These results indicate that endogenous fatty acids may be a key player in the development and differentiation of T cells. Regulatory T cells exhibit an enhanced ability to store lipids in LDs and highly express the enzymes necessary for triacylglycerol synthesis [diacylglycerol acyltransferase (DGAT1&2)] (Howie et al., 2019).

The accumulation of acylated long-chain fatty acids in T cells threatens cell survival. In general, there are three non-mutually exclusive intracellular mechanisms for avoiding the toxic effects of acylated long-chain fatty acids: conversion to triglycerides and storage as LDs, increased β-oxidation in the mitochondria, and removal *via* autophagy (Listenberger et al., 2003; Nguyen et al., 2017). In regulatory T cells, the conversion of diacylglycerols to triacylglycerols has three purposes: lipid storage, avoidance of the toxic effects of acylated long-chain fatty acids, and limitation of the signaling activation of protein kinase C by free diacylglycerol. Foxp3 can upregulate the activity of complexes in the electron transport chain, reprogram and regulate Treg metabolism, drive Tregs to adopt the FAO-OXPHOS metabolism model, and enhance resistance to lipotoxicity (Howie et al., 2017a, 2019). mTOR plays a key role in regulating T cell activation, differentiation, and function, and this ability of mTOR is closely related to its role in promoting T cell metabolic reprogramming. As AMPK and mTOR regulate lipid oxidation and enrich Treg differentiation in the body, inhibition of mTOR and activation of AMPK can lead to increased oxidation of exogenous FAs and promote Treg differentiation (Michalek et al., 2011). Therefore, the availability of metabolic substrates is the driving factor of T cell fate. Instead of using extracellular FAs directly, memory T cells use extracellular glucose to support FAO and oxidative phosphorylation (OXPHOS), indicating that lipids must be synthesized to produce the substrate required for FAO. The inherent lipolysis of cells has a decisive effect on the fate of memory T cells. Memory CD8+ T cells lacking LDs still use inherent lipophilic ER-resident triglycerides for maintenance and function (O'Sullivan et al., 2014). In response to low glucose, naïve T cells and central memory T cells increase oxidative phosphorylation, rely on fatty acid metabolism, increase autophagy, and redirect glutamine to induce fatty acid and pyruvate metabolic pathways (Ecker et al., 2018). The increased dependence on fatty acid oxidation and synthesis after T cell activation is associated with decreased IFN-γ expression. Effector memory T cells, however, do not upregulate fatty acid synthesis and can maintain high levels of IFN-γ production at low glucose levels after T cell activation. In general, different types of T cells sense multiple stresses of lipid metabolism, the translation of which may affect cellular differentiation. When autophagy deficiency occurs, transmission electron microscopy shows the accumulation of LDs in IFT20KD cells, suggesting that T cell autophagy requires IFT20 and that the intraflagellar transporter IFT20 controls lysosomal biogenesis by regulating Golgi posttransport of acidic hydrolases (Finetti et al., 2020).

Overall, T cells rely on different aspects of lipid metabolism to develop, function and differentiate. The heterogeneity of fatty acid metabolic substrates is also closely related to the different functions and phenotypes of T cells (**Figure 6**). The specific mechanisms regarding their regulation and interactions remain unclear.

DISEASES RELATED TO LIPID DROPLETS AND IMMUNE CELLS

Atherosclerosis

Atherosclerosis is one of the leading causes of morbidity and mortality globally. Atherosclerosis is a chronic and progressive inflammatory disease involving the innate and adaptive immune systems that is characterized by endothelial dysfunction, intimal lipid deposition, smooth muscle cell proliferation, apoptosis, and necrosis (Abdolmaleki et al., 2019; Gąsecka et al., 2021). This silent progressive disease, affecting large and mid-sized arteries, with the absence of a luminal elastin barrier and exposure of a dense collagen/proteoglycan, can block blood flow and cause ischemic impairment of downstream tissues or sudden rupture of atherosclerotic plaques (Wolf and Ley, 2019). It has been reported that accumulated lipids represent a central characteristic of inflammatory atherosclerosis and are related to triggering the immune response (Shah, 2019). Thus, this type of disorder exemplifies the connection between abnormal lipid metabolism and immune disease.

The atherogenic process begins with the accumulation of plasma lipoproteins (e.g., low-density lipoprotein, LDL) in the intima, the innermost layer of the artery wall, which experiences flow perturbation and endothelial dysfunction. Vascular wall cells, including smooth muscle cells and endothelial cells, when stimulated by abnormal lipid deposition, mediate leukocyte recruitment and vascular remodeling, as well as the sustained release of proinflammatory cytokines and chemokines. Monocytes/macrophages are also activated due to inflammatory cytokine storms (Ait-Oufella et al., 2011). Differentiated macrophages internalize native and modified lipoproteins (e.g., oxidation resulting in oxLDL) by expressing scavenger receptors and subsequently converting them into cholesterol-rich foam cells, a hallmark of atherosclerosis, leading to a series of complex inflammatory cascades, including developing atherosclerotic lesions, giving rise to fatty streaks that can shape the architecture of advanced plaques. Eventually, the plaque may rupture, causing thrombus formation and possible subsequent infarction of downstream tissue (Weber et al., 2008; Wolfs et al., 2011). Levels of plasma cholesterol, LDL cholesterol, and apolipoproteins are highly correlated with clinical atherosclerosis. Genetic knockout of LDLR (the LDL receptor) or ApoE (apolipoprotein E) in mice, which elevate plasma cholesterol levels, leads to atherosclerosis in C57BL/6 mice (Ishibashi et al., 1993).

Various molecular steps are involved in initiation of the atherosclerotic process. Activation of endothelial cells induces the expression of leukocyte adhesion molecules, such as endothelial-selectin (E-selectin), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin. Migration and infiltration of monocytes are stimulated by the subsequent production of monocyte chemoattractant protein-1 (MCP-1) (Clinton et al., 1992; Gimbrone and García-Cardeña, 2016). Subsequently, monocytes differentiate into macrophages in response to the action of macrophage colony-stimulating factor (M-CSF) (Jones and Ricardo, 2013).

Lipoproteins are modified by oxidizing agents, proteases, and lipases, generating oxLDLs, acetylated LDLs, etc., (Oörni et al., 2000). Macrophages take up oxidized low-density lipoproteins (oxLDL) *via* diverse mechanisms, such as the action of scavenger receptors such as CD36, cholesterol hydrolysis, or phagocytosis, converting to foam cells filled with cytoplasmic LDs, and lipids can be effluxed *via* lipolysis or lipophagy (Chang et al., 1997; Lee and Choi, 2020). C. pneumonia infection results in the chronic inflammatory disease atherosclerosis, leading to lipid droplet-containing foam cell formation within arteries of the host (Libbing et al., 2019). A previous study demonstrated that LDs can be enriched with free cholesterol from hyperlipidaemic serum due to hydrolysis and rearrangement of cellular cholesterol in foam cells (Mori et al., 2001).

The classical categorization of macrophages includes two phenotypes, M1 and M2. Monocytes differentiate into M1 or M2 macrophages in response to exposure to GM-CSF or M-CSF, respectively (Shapouri-Moghaddam et al., 2018). Both types of macrophages are found in atherosclerotic lesions. M1 macrophages are regarded as proinflammatory cells since they secrete various proinflammatory factors, such as TNF-α, IL-1α, and IL-1β, which can recruit inflammatory cells and accelerate plaque development and necrotic core formation, leading to thrombotic events (Chistiakov et al., 2015a). In contrast, M2 (or alternatively polarized) macrophages play an anti-inflammatory and atheroprotective role by restraining cell recruitment and tissue remodeling. They can also reduce the formation of foam cells and strengthen plaque stability. Moreover, M2 macrophages are further subclassified into four subsets (M2a, M2b, M2c, and M2d), depending on their mechanism of activation (Koelwyn et al., 2018).

Both innate immunity and adaptive immunity are crucial for the advancement and expansion of atherosclerosis. The innate response begins with the stimulation of monocytes/macrophages in the vessel walls and is followed by multiple adaptive responses that are modulated by T and B cells. Some effector cells, such as mast cells and eosinophils, may also contribute to atherosclerotic disease (Bot et al., 2007). It has been determined that cell-derived IFN-γ and IL-6 play a proinflammatory role in lesion development. Moreover, eosinophils and activated IgE can promote atherosclerosis progression (Xin et al., 2020). From a disease treatment perspective, immune modulation of T and B cell-mediated responses represents an attractive antiatherosclerotic therapeutic strategy.

Mycobacterium tuberculosis

Mycobacterium tuberculosis (M.tb), the causal agent of tuberculosis and the leading cause of death among individuals living with HIV, is likely the most successful human pathogen. The interplay between M.tb and lipid metabolism and the host immune response creates a complex pathogen-host interaction

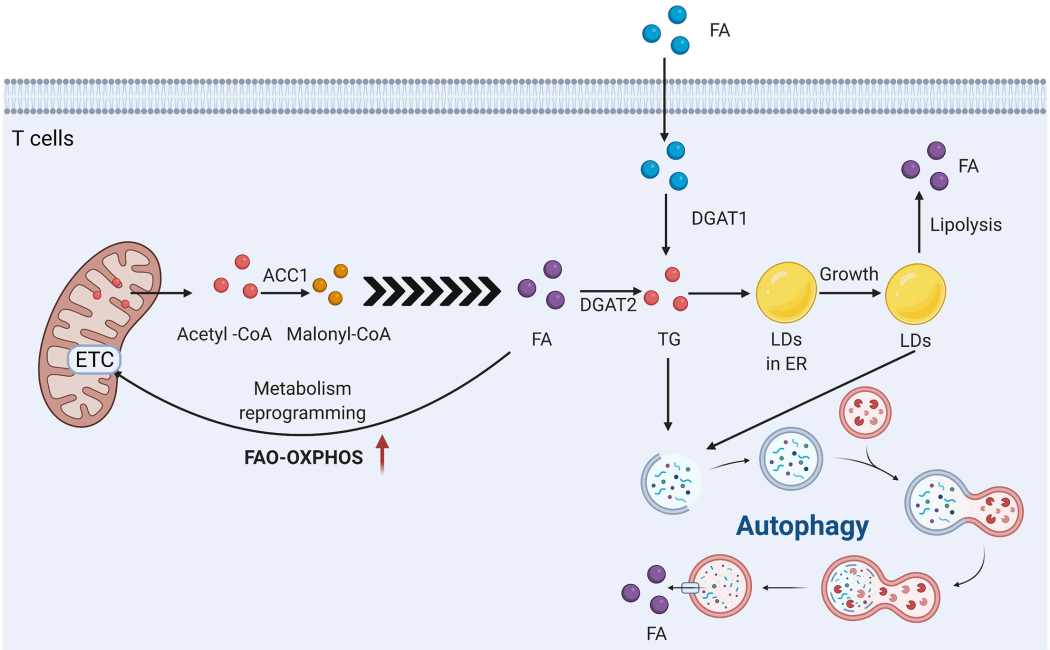

FIGURE 6 | T cells rely on the different aspects of lipid metabolism to develop function and differentiation. Acetyl-Coenzyme A carboxylase 1 (ACC1) is the key for the fatty acid generation in T cells. It can convert Acetyl-CoA into Malonyl-CoA. LDs are derived from the endoplasmic reticulum, and DGAT1 and 2 esterify free acylated fatty acids to diacylglycerols to form more inert triacylglycerols, which are stored in the LDs core. DGAT1 is responsible for the esterification of mainly exogenous fatty acids into TG, while DGAT2 is considered to have advantages in the esterification of endogenous synthetic FA. In general, there are three non-mutually exclusive intracellular mechanisms to avoid the toxic effects of acylated long-chain fatty acids: conversion to triglycerides and storage as LDs, increased β-oxidation in the mitochondria, and removal *via* autophagy.

(Bailo et al., 2015). In particular, lipids serve as the key mediators of this interaction, not only as nutrient sources for M.tb but also as modulators of host immune responses. Interestingly, this bacterium seems to preferentially utilize host lipids to maintain self-metabolic activity (Pandey and Sassetti, 2008). Moreover, there are specific host pathways, such as the PPARγ and LXR transcriptional regulators, that have revealed the mechanisms by which host immunity alters the bacterial microenvironment. One hallmark of tuberculosis infection is the accumulation of M.tb-infected foamy macrophages containing large lipid bodies (Wong et al., 2020). In this review, we focus on emerging concepts and the current understanding of lipid utilization in M.tb, how the immune response modulates lipid homeostasis in both the bacterium and the host, and how these pathways are likely to be manipulated therapeutically.

As a highly successful intracellular pathogen, M.tb can tolerate harsh conditions and reside long-term within its host by refining its metabolism. Studies conducted over the past 60 years have clearly shown that M.tb relies on fatty acids and cholesterol as important nutrients during infection (Bloch and Segal, 1956; Chang and Guan, 2021). There are two types of growth behavior in bacteria: diauxic and coutilization of carbon sources. M.tb has a preference for the latter, and it can catabolize multiple carbon sources simultaneously through compartmentalization of separate metabolic processes (de Carvalho et al., 2010).

Fatty acids rather than carbohydrates are the primary carbon source for M.tb during infection. M.tb utilizes fatty acids for specific purposes, including as substrates for beta-oxidation, acyl primers for polyketide lipid synthesis, or by incorporating them into phospholipids and/or triacylglycerol (TAG) (Lovewell et al., 2016). Most importantly, M.tb β-oxidizes fatty acids to produce energy in its crucial central metabolism. Second, fatty acids are also donated to the production of virulence-associated molecules, such as polyketide lipids phthiocerol-dimycocerosate (PDIM), polyacylated trehalose (PAT), sulfolipid (SL), and mycolic acids. Third, fatty acids can be assimilated directly into cell membrane phospholipids, which maintain cytoplasmic membrane integrity or are converted into cellular TAG, which functions as a carbon storage molecule (Trivedi et al., 2004; Daniel et al., 2011; Baran et al., 2020).

Several lines of evidence indicate that lipid metabolic pathways not only serve as a source of carbon and energy but can also protect M.tb from stress. For example, TAG synthesis reduces the M.tb growth rate and antibiotic sensitivity by reducing acetyl-CoA availability. Cytosolic redox can be influenced through the balance of fatty acid catabolism and anabolism (Jansen et al., 2020). Host lipids have been shown to play a dominant role in optimal growth and persistence of M.tb during infection. One possibility for the mechanisms of acquiring host lipids is that fatty acids from LDs or lipid bilayers originate from the host cell for use by M.tb. Another possibility is that M.tb acquires cholesterol and fatty acids directly from lipoproteins within the macrophage endocytic network trafficking in a soluble form (Bonds et al., 2020).

During tuberculosis infection, the immune response of the host can cause a variety of changes in both intracellular and extracellular bacteria. Coordinated efforts of the innate and adaptive immune systems are required (Crowther and Qualls, 2020). Inflammatory signals triggered by many microbial pathogens, such as M.tb and Chlamydia pneumoniae, induce the accumulation of LDs in immune cells, such as neutrophils, eosinophils, and macrophages. Macrophages then adopt a foamy morphology and thus are termed foam cells. Interestingly, in foam macrophages, mycobacteria are present with phagosomes that are very close to LDs and transition to a dormant state. Moreover, the accumulation of lipids by M.tb within foam macrophages is primarily a result of the incorporation of fatty acids derived from host TAG in a process largely mediated by mycobacterial triacylglycerol synthase 1 (Maphasa et al., 2020).

Several clarified mechanisms underlie immune-mediated alterations in macrophage lipid homeostasis. One major mechanism behind this modulation involves the infection-dependent activation of peroxisome proliferator-activated receptors (PPARs). M.tb infection can increase PPAR-γ expression in infected cells in a Toll-like receptor 2 (TLR2)-dependent manner, and activation of these signaling pathways leads to lipid droplet accumulation. Furthermore, host-cell lipid signaling programs such as the CD36 pathways are now being revealed as important routes for determining outcomes during M.tb infection. Finally, lipid accumulation in the M.tb-infected host could exert additional effects on the pathogen. It not only provides a growth substrate for the bacterium and promotes a hyperinflammatory environment but also increases the intracellular lipid pool, which can trigger an anti-starvation response and inhibit autophagy and lysosome acidification (Parihar et al., 2014; Paik and Jo, 2020; Chen et al., 2021).

Cancer

Although prominently found in adipose tissue, LDs can exist in all cell types and tissues (Lynch and Scott, 1951). Higher LD contents have been reported in cancer cells and cancerous tissues such as colorectal cancer, breast and prostate cancers, hepatocellular carcinoma, renal cell carcinoma and glioblastoma, and LDs can extensively mediate proliferation, invasion, metastasis and chemotherapy resistance in various cancers. In this article, we summarized the indispensable role that LDs play in carcinogenesis, malignant development of cancers, and immune cells.

Lipid metabolic enzymes are responsible for the synthesis or degradation of LD components, such as LPCAT2, cytosolic phospholipase 2 (cPLA2), sterol *O*-acyltransferase 1 (SOAT1), etc. Sterol regulatory element-binding protein-1 (SREBP-1) is a key transcription factor in regulating lipid synthesis and uptake. Recent studies have revealed that SREBPs are highly activated in various cancers and promote tumor growth, which shows promise for potential drugs targeting SREBP-1-driven lipid metabolism as anticancer agents (Brown and Goldstein, 1997). In addition, LD coat proteins (Perilipins) and fatty acid-binding proteins (FABPs) are also involved in the regulation of LD formation and trafficking in cancer cells. A-FABP expression in macrophages promotes breast cancer growth and metastasis through IL-6/STAT3 signaling, whereas E-FABP expression in macrophages strengthens type I interferon β (IFN β) responses against tumor progression (Zhang et al., 2014; Hao et al., 2018).

Lipid droplets are composed of a monolayer of phospholipids as well as a hydrophobic core of neutral lipids (consisting of primarily triacylglycerols and cholesterol esters). Increased storage of lipids in LDs is beneficial for the malignant development of cancers. This was illustrated by a study in a breast cancer cell line, where LD abundance was shown to correlate with the degree of invasiveness from non-malignant MCF10A cells to highly malignant MDA-MB-231 cells (Nieva et al., 2012). Intracellular excess fatty acids and cholesterol stored in LDs prevent lipotoxicity and endoplasmic reticulum stress. Moreover, increased LD contents could expand the source of lipid substrates and energy to support the metabolic requirement of proliferative cancer cells. For example, in the tumor microenvironment (TME), LDs could provide energy for aggressive cancer to trigger metastatic cloning. The lipid-rich tumor microenvironment also resulted in defective antitumor properties in dendritic cells (DCs). DCs, a type of professional antigen-presenting cell, can initiate and sustain T cell-dependent anticancer immunity. A previous study found that DCs with high LD content were unable to present tumor-associated antigens or stimulate allogeneic T cells in tumor-bearing mice. The accumulation of LDs was caused by increased uptake of extracellular lipids (Veglia et al., 2017). Another study verified that the abnormal build-up of LDs in tumor-associated DCs could be activated by the intrinsic ER stress-dependent XBP1 pathway, which promoted ovarian cancer progression (Cubillos-Ruiz et al., 2015). The effects of lipid regulation on DC function in cancer were studied by using 5-(tetradecyloxy)-2-furoic acid (TOFA) to suppress lipid accumulation, which led to restoration of the functional capacity of DCs and enhanced the activity of the antitumor T-cell response (Herber et al., 2010). The molecular mechanisms underlying defective antigen presentation by high LD content remain to be elucidated. LD accumulation in cancer cells may modulate the effectiveness of antitumor immune responses and therefore provide a promising therapeutic target.

CONCLUSION

In recent years, LDs have received worldwide attention, and breakthrough results have been achieved in many fields, especially with respect to the contribution of LDs to the immune system. With the popularity and gradual development of the concept of metabolic immunity in recent years and the characteristics of LDs as a key intermediate bridge connecting metabolism and immunity, the role and specific mechanism of LDs in immune and metabolic diseases have been revealed by an increasing number of studies, which have greatly improved and supplemented understanding of the functional and structural characteristics of LDs. Although many studies have been conducted on the biogenesis of LDs, lipid degradation, and the physical and functional connections between LDs and other organelles in the body, many mechanisms and details are still unknown and need to be further explored. Lipid drops are

closely related to the immune system, especially the synthesis and secretion of some cytokines of lipid bioactivity, which are regulated by lipid drops. Previously, it was thought that LDs support infection by providing substrates to synthesize membrane and produce energy, ensuring the stability of the pathogen membrane structure and the persistent development of infection. However, recent research results seem contradictory. They assert that in response to infection, mammalian fat droplets assemble antibiotics and immune proteins to form a complex that fights pathogen invasion. Thus, the anti-infection effect of LDs on the immune system remains controversial. One possible explanation for this controversy is that the function of LDs is variable and may be related to the organism, tissue, cell, subcellular location, etc. Similar to the functional and morphological heterogeneity of mitochondria in different microenvironments, as a kind of organelle, whether the heterogeneity of LDs is not limited to their number, size, and composition and whether their function dynamically displays different characteristics, depending on the microenvironment in which they are located remain to be further explored. Although the strong correlation between LDs and the immune system plays an encouraging role in atherosclerosis and anti-TB bacilli, a serious reality is that there is still a lack of safe and effective therapeutic targets that can be applied in clinical practice at present. This partly explains why the connection between them still has much uncertainty. Therefore, a thorough understanding of the role of LDs in the immune system and the mechanism of regulating immune cells is of great significance for the development of new anti-infection drugs and the improvement of therapeutic targets for atherosclerosis.

AUTHOR CONTRIBUTIONS

MZ and LX: conceptualization. WZ: writing—original draft preparation. WZ, LZ, and LX: writing—review and editing. WZ and SY: visualization. MZ: supervision. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Lipids as Regulators of Cellular Senescence

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Lipids are key macromolecules that perform a multitude of biological functions ranging from maintaining structural integrity of membranes, energy storage, to signaling molecules. Unsurprisingly, variations in lipid composition and its levels can influence the functional and physiological state of the cell and its milieu. Cellular senescence is a permanent state of cell cycle arrest and is a hallmark of the aging process, as well as several age-related pathologies. Senescent cells are often characterized by alterations in morphology, metabolism, chromatin remodeling and exhibit a complex pro-inflammatory secretome (SASP). Recent studies have shown that the regulation of specific lipid species play a critical role in senescence. Indeed, some lipid species even contribute to the low-grade inflammation associated with SASP. Many protein regulators of senescence have been well characterized and are associated with lipid metabolism. However, the link between critical regulators of cellular senescence and senescence-associated lipid changes is yet to be elucidated. Here we systematically review the current knowledge on lipid metabolism and dynamics of cellular lipid content during senescence. We focus on the roles of major players of senescence in regulating lipid metabolism. Finally, we explore the future prospects of lipid research in senescence and its potential to be targeted as senotherapeutics.

Keywords: cellular senescence, lipids, aging, bioactive lipids, senolytics

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INTRODUCTION

Senescence is a cell-fate decision that is triggered in response to many different stressors including genotoxic stress, telomere attrition/damage, oncogene activation, and mitochondrial dysfunction (Herranz and Gil, 2018). In contrast to quiescence (cell cycle arrest), cellular senescence is a terminal state of growth arrest, where cells cannot re-enter the cell cycle despite mitogenic growth signals (Pack et al., 2019). Senescent cells resist apoptosis, at least partially due to an upregulation of survival pathways such as the BCL-2 family (Zhu et al., 2015; Yosef et al., 2016). Cellular senescence has been documented to have both beneficial and detrimental roles in maintaining health (He and Sharpless, 2017), and has been linked to embryonic development, wound healing, tumor suppression, as well as metastasis and aging. "Acute" senescence is a programmed cell-fate decision and is usually associated with tissue repair and homeostasis (Krizhanovsky et al., 2008; Jun and Lau, 2010; Rajagopalan and Long, 2012). In contrast, "chronic" senescence is associated with stochastic, persistent macromolecular damage and often associated with disease and aging (van Deursen, 2014). The permanent cell-cycle arrest of senescent cells is accompanied by a senescence associated secretory program (SASP). The SASP consists of pro-inflammatory factors such as cytokines, chemokines and extracellular matrix remodeling factors.

IDENTIFICATION OF SENESCENT CELLS

The field is challenged by the lack of a single universal marker for cellular senescence. Currently, senescent cells are identified based on multiple markers (**Figure 1**), that allow for distinction between quiescent and senescent cells. The most prominent features of senescent cells include, an enlarged, flat cell morphology with an increase in the cytoplasm-to-nucleus ratio. A recent study suggested that the ratio of DNA to cytoplasm is critical to maintain cell function, and dysregulation of this ratio possibly contributes toward aging (Neurohr et al., 2019). Senescent cells also display an increase in the lysosomal enzyme, senescence-associated-β-galactosidase (SA-β-gal), and thus display increased SA-β-gal activity (Dimri et al., 1995; Hall et al., 2017). Single cell analysis, identified that cells with high SA-β-gal activity also exhibit larger cell size (Biran et al., 2017). It is important to note that SA-β-gal is often associated with senescent cells, but can be observed upon serum starvation or upon confluency in cell culture. Another feature of senescent cells is the accumulation of pigment granules in the lysosomes known as lipofuscin (Evangelou et al., 2017). However, lipofuscin accumulation is observed with age in many cells and organisms, including those that do not typically have other features of cellular senescence, e.g., *C. elegans* (Pincus et al., 2016). Lack of DNA replication and arrest of the cell cycle usually in G0/G1 phase are features of senescent cells, but do not distinguish between quiescence and senescence.

The two key signaling pathways upregulated for establishment and maintenance of senescence are p53/p21 and p16 (discussed in detail below). The burden of p21- and p16- positive expressing cells increases with age in mammals, however, currently the understanding of what percentage of cells are senescent is incomplete. A recent cross-sectional study in humans, examining three different age groups and multiple tissues, identified that p21- and p16-positive cells increase with age in the skin, pancreas and kidney (Idda et al., 2020). In contrast, p16-positive cells are observed in brain cortex, liver, spleen, and intestine (colon), whereas p21-positive cells increase in the dermis with age. Interestingly, some tissues, such as lung and muscle did not show any changes in these markers. Although a comprehensive study, it is important to note that this study was cross-sectional in design, as well as underlying health conditions may not have been fully examined. Nonetheless, a conclusion to be drawn from this study is that different cells and tissues accumulate different markers of senescence and may display varying burden of senescent cells.

Dynamic chromatin changes are a hallmark of senescent cells. Senescence-associated heterochromatin foci (SAHF) and telomere-associated foci (TAFs) are commonly used as markers for cellular senescence. However, these heterochromatin changes have been associated with senescence caused by nuclear DNA damage or telomere dysfunction, rather than other senescence-inducing stressors (Di Micco et al., 2011). A common chromatin feature in senescent cells is the generation of chromatin fragments that are released into the cytoplasm. The loss of nuclear lamina (Lamin B1) is observed in senescent cells, leading to "leaky" nucleus, and is thought to promote chromatin fragments (CCFs) in the cytoplasm (Di Micco et al., 2011; Dou et al., 2015, 2017). The CCFs activate the cyclic GMP–AMP synthase (cGAS) and the stimulator of interferon genes (STING) pathway (cGAS-STING pathway) and further establish SASP factors (Vizioli et al., 2020), thus reinforcing senescence and affecting neighboring cells.

Last but not least, an integral part of senescent cells is their ability to drive the SASP phenotype. SASP consist of pro-inflammatory cytokines [interleukin (IL)-1α, IL-1β, IL-6, and IL-8], chemokines (CCL2), and extracellular matrix remodeling factors such as matrix metalloproteinases (MMPs), serine/cysteine proteinase inhibitors (SERPINs) such as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) (Coppe et al., 2010; Eren et al., 2014). With advances in technology, recent studies have uncovered that different senescence-inducing stressors, as well as different cell types may lead to unique SASP being expressed (Basisty et al., 2020). SASP is thought to be transcriptionally controlled and released into the cytoplasm, as soluble factors or in exosomes (Lehmann et al., 2008; Ozcan et al., 2016; Takahashi et al., 2017), to influence neighboring cells (Ito et al., 2017). Understanding of SASP regulation is incomplete, although, p53, NF-κB, P38 MAPK, NOTCH, mTOR, and mitochondrial NAD+/NADH ratio have recently emerged as modulators of the SASP phenotype (Freund et al., 2011; Herranz et al., 2015; Nacarelli et al., 2019).

In this Review, we first describe the rewiring of lipid metabolism during cellular senescence. This review mostly focuses on cellular senescence observed with aging. We then review the link between major regulators of senescence and their role in lipid metabolism. Lastly, we discuss lipid classes that are altered in senescent cells and how they contribute to the senescence phenotype and outline tools that can help examine the causal role of lipids in driving cellular senescence.

METABOLIC LANDSCAPE OF SENESCENT CELLS

Aging is associated with dysregulated lipid metabolism (Mutlu et al., 2021). Several studies show that lipid composition changes with age in several species, including humans. Particularly, increased ratio of poly- and mono- unsaturated fatty acids have been recently found to be increased with age (Papsdorf and Brunet, 2019). However, whether cellular senescence, a key feature of aging is responsible for these lipid changes has been enigmatic so far. Lipids are an important fuel source that generate energy, as well as create crucial biological intermediates including lipid mediators, second messengers, and ketone bodies. Lipids can act as central metabolites that play a role in cellular signaling, both in an autocrine and paracrine manner, provide cell and organelle structure, and are fundamental for maintaining cellular homeostasis. Recent studies highlight the role of lipids in establishing cell fate decisions, such as cell division, apoptosis and cellular senescence (Ito and Ito, 2016). As described above, hallmarks of cellular senescence consists of increased mass of organelles such as, lysosomes and mitochondria, increased cellular size, loss of nuclear membrane integrity and SASP

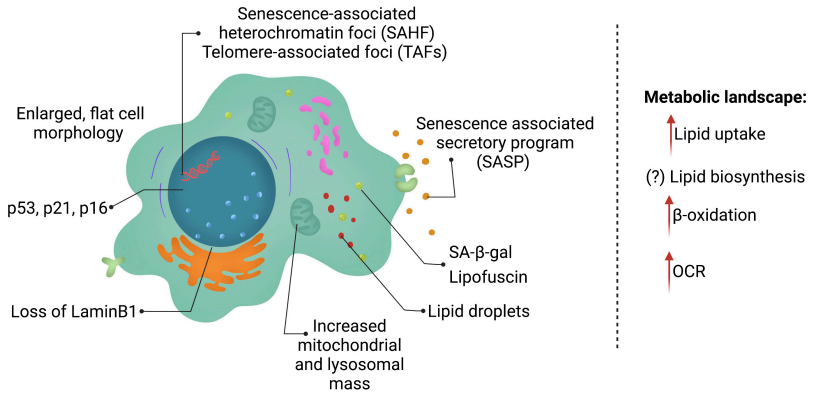

FIGURE 1 | Features of senescent cells. Senescent cells are in a state of permanent cell cycle arrest and display several key features including lysosomal changes (SA-β-gal and lipofuscin), p53, p21, and/or p16 expression and a SASP program consisting of multiple cytokines, chemokines, and ECM remodeling enzymes. Senescent cells reprogram their metabolism as well heavily altering lipid metabolism.

factors that can be "transported" by exosomes (Lizardo et al., 2017). Lipids are essential to each of these "senescence features," including exosomes that are typically lipid enriched extracellular vesicles (Skotland et al., 2019) and therefore lipids are inextricably linked to the process of cellular senescence.

Several studies report an accumulation of lipid droplets in senescent cells compared to proliferating cells (Ogrodnik et al., 2014, 2019; Cox and Redman, 2017; Flor et al., 2017; Lizardo et al., 2017; Chee et al., 2021). Deregulated lipid accumulation in senescent cells can be due to increase in lipid uptake, upregulated lipid biosynthesis pathways or de-regulated lipid breakdown. Interestingly, senescent cells display increased lipid uptake (Flor et al., 2017), however, whether inhibition of lipid uptake can prevent or delays cellular senescence is unclear. Lipid levels are also controlled through a complex mechanism of lipid biosynthesis and breakdown. Advances in technology such as non-targeted "omic" approaches have highlighted the importance of lipid metabolism in cellular senescence. For example, a recent proteomic study of senescent cells, induced by DNA damaging agents, uncovered that lipid processing and metabolism genes including those involved in lipid binding, storage, biosynthesis and breakdown are severely altered (Flor et al., 2017). Fatty acids (FA) are essential building blocks for triglycerides (TGs) and phospholipids, and act as an energy source. Fatty acids are synthesized by the addition of malonyl-CoA to an acyl chain by one of two enzymes: acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). Although understudied, the role of FA biosynthesis in cellular senescence is possibly regulated in a context- and stress-dependent manner. Enlargement of cells and accumulation of membranous organelles such as, lysosomes and mitochondria are key hallmarks of senescence, and rely on lipid biosynthesis (Kim et al., 2010; Correia-Melo et al., 2016). Consistent with this theory, mouse hepatic stellate cells and human primary fibroblasts showed an increase in lipid biosynthesis, dependent on FASN, during initial establishment of cellular senescence (Fafian-Labora et al., 2019). FASN is upregulated prior to induction of late-stage senescence, and is increased with age in mouse liver. Interestingly, pharmacological or genetic inhibition of FASN, reduced p53-induced senescence, as well as, SASP (Borghesan et al., 2019; Fafian-Labora et al., 2019). Whether FASN is required for maintenance of cellular senescence and upregulated in a p53-independent manner is currently unknown. Oncogene-induced senescence (OIS) is a robust mechanism activated by oncogenic signaling due to an activating mutation of an oncogene or an inactivation of a tumor suppressor. Targeted metabolomic and bioenergetic analysis revealed that different modalities of senescence such as OIS and replicative senescence, led to very different metabolite patterns. In this study, Ras-induced OIS elevated levels of long chain free fatty acids (LCFA). Concomitantly, OIS reduced lipid biosynthesis by negatively regulating ACC, a rate limiting step for lipid biosynthesis (Quijano et al., 2012). It is important to note that these contradictory results about the role of lipid biosynthesis in senescence may be due to analyzing a different "stage" of cellular senescence, or different stress modality of senescence (aging versus OIS) or because of differences in cell-type (primary cells versus cancer cells).

Although the role of lipid biosynthesis in cellular senescence may not be straight-forward, FA breakdown has been consistently observed during senescence. A few reports indicate that lipid breakdown particularly through mitochondrial β-oxidation plays a paramount role in cellular senescence. Increased mitochondrial function has long been associated with senescence. Fatty acid oxidation (β-oxidation) is the mitochondrial multi-step process of breaking down a fatty acid into acetyl-CoA units. Long chain fatty acids (LCFA) are activated by acyl-carnitines and imported into the mitochondria for β-oxidation via a rate limiting enzyme carnitine palmitoyltransferase (CPT). Upon completion of β-oxidation, the generated acetyl-CoA can enter the TCA cycle, link to oxidative phosphorylation (OXPHOS) or can lead to acetylation of histones and other proteins. Indeed, recent data suggests that lipids contribute heavily toward histone acetylation and control gene transcriptional programs (McDonnell et al., 2016). During replicative senescence in myoblasts, several acyl-carnitines were increased, suggesting that senescent cells rely

heavily on β-oxidation (Baraibar et al., 2016). Additionally, these senescent myoblasts had increased levels of monoacyl glycerols and glycerol-3-phosphate, as well as decreased mono- and polyunsaturated long chain fatty acids (>18 carbons), whereas medium chain fatty acids remained unaffected. This suggests that senescent cells increase lipid breakdown, however, whether this lipid mobilization and breakdown is used for energy production, gene transcription or generation of SASP (bioactive lipids) is unclear. Interestingly, an increase in FASN during establishment of the senescence program is also associated with an increase in CPT function and oxygen consumption rate (OCR). Consistent with this, inhibition of CPT1A during OIS, prevented cellular senescence and SASP establishment (Quijano et al., 2012). Collectively, these studies suggest that β-oxidation is required to drive cellular senescence (Fafian-Labora et al., 2019). It is important to note that one study reported that inhibition of β-oxidation could possibly promote senescence (Baraibar et al., 2016), Here, loss of CPT1C in lung fibroblasts, promoted lipid accumulation in cells and induced cellular senescence through lipotoxicity. One possibility is that lung fibroblasts uniquely depend on lipid β-oxidation, and inhibition of this process drives lipotoxicity and cellular senescence (Chen et al., 2021). Overall, these studies point toward major rewiring of lipid metabolism during establishment and maintenance of cellular senescence.

LIPID COMPOSITIONAL CHANGES DURING CELLULAR SENESCENCE

Although several studies show accumulation of lipid droplets (LDs) during cellular senescence, the content of these LDs may be far more critical. Lipids are divided into three groups, triglycerides (TAGs) (utilized for energy), steroids (cholesterol and hormones) and phospholipids (utilized for membrane structures). Here, we summarize the associated changes in composition of these lipids during senescence.

Triglycerides

A study to decipher the transcriptomic and lipidomic changes during replicative senescence showed that lipid uptake mediated by CD36 is upregulated in senescent cells (Saitou et al., 2018). This feature is also consistent with DNA damage, oncogenic and chemical stress-induced senescence (Lizardo et al., 2017; Chong et al., 2018). Moreover, there is an enrichment of TAGs containing long- and very long-chain fatty acids, with at least one polyunsaturated fatty acyl (PUFA) chain (Saitou et al., 2018). Another study reported that DNA damage-induced senescence is accompanied with the upregulation of the triglyceride synthesis enzyme, diacylglycerol acyltransferase 1 (DGAT1). This study suggests that rather than lipid uptake, TAG biosynthesis may play a crucial role in establishment of senescence (Flor et al., 2017). The caveat though is that in this study the TAG species were not directly measured. It is therefore possible that both uptake and biosynthesis of TAGs may play a role in promoting cellular senescence.

Cholesterol

Cholesterol homeostasis is critical to health. It is an important building block for hormones, vitamin D and integral to cell membrane integrity; however, excess cholesterol can have detrimental effects. Few studies have examined the role of cholesterol on cellular senescence. Naked mole rats (NMR) are known for their exceptional longevity, resilience to endogenous and exogenous stressors and cancer resistance. Interestingly, NMR fibroblasts had elevated levels of cholesterol-enriched lipid droplets (LDs), dependent on the Wnt/β-catenin pathway (Chee et al., 2021). Inhibition of cholesterol synthesis using lovastatin, an HMG-CoA reductase inhibitor, promoted senescence-like phenotype in NMR fibroblasts. Of note, only SA β-gal and 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker for DNA damaged by oxidative stress was measured in this study. Furthermore, the Wnt/β-catenin-cholesterol axis seems specific to NMR, as overexpression of β-catenin in mice or rat fibroblasts did not show any elevation in LDs (Chee et al., 2021). On the contrary, lipid profiling of OIS induced by ERBB2 (a member of the epidermal growth factor receptor), revealed increased cholesterol levels accumulated in vacuoles of senescent cells (Cadenas et al., 2012). This study suggests that high cholesterol levels are possibly a feature of senescence, although whether this accumulated cholesterol is a driver of senescence is not fully understood. In contrast, when mouse bone marrow stem cells (BMSCs) were exposed to low dose of cholesterol, it delayed oxidative stress-induced cellular senescence. Indeed, low levels of cholesterol promoted proliferation of BMSCs. Oxidative stress is known to damage macromolecular structures including cellular membranes, and since cholesterol is a major component of cell membranes, exogenous supplementation of cholesterol may help recovery from acute damage (Zhang et al., 2016). Further studies to examine the impact of cholesterol dosage on senescence *in vivo* is required to ascertain its exact role in the senescence phenotype.

Phospholipids

Phospholipids are amphiphilic lipids that play important metabolic and structural roles. In a study looking at ERBB2 induced senescence (OIS), phospholipid with shortened acyl chains and unsaturated acyl chains in phosphatidylglycerol was observed (Cadenas et al., 2012), accompanied by increased membrane fluidity. This study also identified that mitochondrial lipids were altered, specifically, PG(34:1), PG(36:1) (increased) and LPE(18:1), PG(40:7) and PI(36:1) were reduced in senescent cells leading to loss of membrane potential. A comprehensive study compared and analyzed metabolites in replicative senescence, DNA damage-induced senescence and OIS in a human embryonic lung fibroblast (WI-38), using NMR spectroscopy. Interestingly, senescent cells displayed increased glycerophosphorylcholine-to-phosphocholine ratio. Importantly, glycerophosphorylcholine levels were particularly elevated in senescent cells compared to proliferating or quiescent cells, suggesting that this could be a unique feature of senescence (James et al., 2015). Bioactive phospholipids play an important role in "communication" and consist of

lysophospholipids, ceramides, and sphingomyelin (Horn and Jaiswal, 2019). They can be released as secreted molecules or part of exosomes, and possibly play a role in extravesicular formation and release observed during senescence (Tepper et al., 2000; Nurminen et al., 2002; Trajkovic et al., 2008; Bianco et al., 2009; Hirsova et al., 2016). Hydrolysis of phospholipids by phospholipases, such as cPLA2, leads to lysophospholipids. An increase in lysophospholipids has been observed in senescent cells (Buratta et al., 2017; Narzt et al., 2021), and furthermore, lysophospholipids are shown to induce senescence in cholangiocytes (Shimizu et al., 2015). They can also act as a "find me" signal, but impair phagocytosis, possibly leading to inefficient clearance of senescent cells with age (Narzt et al., 2021). Phospholipids also influence the synthesis of eicosanoids that are known inflammatory mediators, thus contributing to the SASP phenotype (discussed in detail below). Thus, phospholipids play a varied role during senescence, including establishment of senescence, paracrine signaling and evasion of senescent cells.

MAJOR LIPID PLAYERS IN SENESCENCE

Oxidized Fatty Acids

One of the major components of complex lipids in mammalian cells are polyunsaturated fatty acids (PUFAs) with atleast one bis allelic carbon and chain lengths ranging from 18 and above. PUFAs are precursors for potent metabolites that are involved in numerous pathophysiological events. One of the most well-studied families are eicosanoids, a class of oxygenated derivatives of arachidonic acid (C20) that include prostaglandins, leukotriens, lipoxins, hydroxy eicosatetraenoic acids, hydroperoxy eicosatetraenoic acids, and epoxy eicosatetraenoic acids. These bioactive lipids are derived enzymatically through the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) reactions or non-enzymatically through free radical reactions. Together, these bioactive lipids regulate diverse sets of both homeostatic and inflammatory processes. Pharmacological inhibition of the cysteinyl leukotrienes via LT antagonists, LY255283 and montelukast sodium, or ALOX5 inhibitor, BW-B70C, reduced the profibrotic effect of senescent cells on naive fibroblasts *in vitro* (Wiley et al., 2019) and attenuated TNFα-induced up-regulation of SA-β-Gal, p53, p21, and PAI-1 expression in primary human chondrocytes associated with osteoarthritis (Song et al., 2018). Increase in COX-2 activity has been shown to be associated with aging (Badawi et al., 2005; Chung et al., 1999; Baek et al., 2001). For example, COX-2 expression was upregulated in kidney, liver, heart and prostrate in old rodents (Hayek et al., 1997; Kim et al., 2001; Badawi et al., 2004; Choi et al., 2014; Tung et al., 2015), and in aging human tissues as well, especially kidney and skin (Melk et al., 2004; Kang et al., 2006; Surowiak et al., 2014). Although the cause- or-causal relationship is not clear, it was shown that in an inducible *Cox-2* transgenic mice, post-natal expression of COX-2 led to several premature aging phenotypes (Kim et al., 2016). Further, the lung fibroblasts from these animals expressed higher levels of senescence-associated SA-β-Gal, p16, p53, and phospho-H_2Ax (Kim et al., 2016).

The age-related changes in COX-2 are often accompanied by an elevation in the prostaglandin synthesis. COX system is the major pathway catalyzing the conversion of arachidonic acid into prostaglandins, PGG2 and subsequently to PGH2 followed by the production of the bioactive lipids—PGE2, PGI2, PGD2, PGF2α—and thromboxane A2 by tissue-specific synthases. An upregulation in COX-2 expression and ensuing PGE2 production was observed during normal and stress-induced senescence of dermal, prostrate and lung human fibroblasts. Exogenous supplementation of arachidonic acid enhanced COX-2 activity and PGE2 production and subsequently accelerated the incidence of key senescence features like flat and enlarged cell morphology, SA-β-Gal activity and the cell cycle arrest (Dagouassat et al., 2013). In a study to understand the mechanisms of lung senescence linked with chronic obstructive pulmonary disease (COPD), it was shown that prostaglandin E2 released by pulmonary fibroblasts exerted both autocrine and paracrine effects to induce fibroblast senescence. This was accompanied with inflammation by EP2 and EP4 receptors, COX-2–dependent reactive oxygen species signaling (Dagouassat et al., 2013). Wiley et al. recently showed that senescent cells produced and accumulated 1a,1b-dihomo-15-deoxy-delta-12,14-prostaglandin J2, which together with other prostaglandin D2-related lipids promoted senescence arrest and SASP through activation of RAS signaling. In mice with drug-induced senescence, a dose of Navitoclax, a senolytic drug (eliminates senescent cells) elevated the levels of prostaglandin in blood and in urine. This study highlights the importance of prostaglandin lipids as biomarkers of senolysis.

Another primary oxidation product of arachidonic acid is hydroperoxy-eicosatetraenoic acid (hydroperoxyeicosatetraenoic acid, HpETE), generated via lipoxygenase (LOX) activity. Due to its regiospecificity nature, lipoxygenases are classified as arachidonate 5-, 8, 12-, and 15-lipoxygenase (5-LOX, 8-LOX, 12-LOX, and 15-LOX), which inserts oxygen at carbon 5, 8, 12, or 15 of arachidonic acid peroxidases. The primary oxidation products of HpETE are unstable and reduced further to its corresponding alcohol hydroxy-eicosatetraenoic acid (5-, 8-, 12-, and 15- hydroxy-6-trans-8,11,14-cis-eicosatetranoic acid, HETE) by glutathione peroxidase. The enzyme 5-LOX can act on 5-HETE to generate an unstable epoxide leukotriene A4 (LTA4), which can serve either as an intracellular intermediate in the synthesis of leukotriene B4/C4 (LTB4 and LTC4). Leukoterines (LTs) are the second major class of biologically active signaling lipids that plays a key role in inflammation and fibrosis. Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease with elevated senescent cell burden. It was shown that leukoterines secreted by senescent cells exacerbated IPF by promoting pulmonary fibrosis (Wiley et al., 2019) and kidney fibrosis by unilateral ureteral obstruction in mice (Kamata et al., 2019). Senescence induced irrespective of different modalities (oncogene-, stress-, and radiation-induced) in IMR-90 fibroblasts promoted secretion of leukoterines, as well as expression of enzymes involved in the biosynthesis of LTs (Wiley et al., 2019). Cysteinyl leukoterines such as LTC4 has been shown to accelerate

acute kidney injury and drive renal pathogenesis in response to chemotherapy-induced senescence (Rubinstein and Dvash, 2018; Wiley, 2020). In line with these findings, leukotrienes increasingly released from senescent cells, irrespective of the senescence-inducer, and appear to function in parallel to SASP factors.

Besides lipoxygenases and cyclooxygenases, the third class of enzymes involved in eicosanoid synthesis are epoxygenases or cytochrome P450 (CYP) enzymes. Cytochrome P450 epoxygenases (CYP2C and CYP2J) synthesize epoxides, such as epoxyeicosatrienoic acid (EETs) in four different regioisomers (5, 6-, 8, 9-, 11, 12-, and 14,15-) that are recognized as specialized pro-resolving mediators (Alkayed et al., 1996; Zhang et al., 2007; Li et al., 2012). EETs have a wide range of protective actions such as anti-inflammatory, anti-apoptotic, vasodilatory and pro-angiogenic (Node et al., 1999; Iliff et al., 2009). The levels of EETs are often determined through production of dihydroxyeicosatrienoic acid (DHET) isoforms via soluble epoxide hydrolase (sEH) (Morisseau and Hammock, 2013). Gene deletion of soluble epoxide hydrolase was shown to improve blood flow and reduce infarct size following cerebral ischemia in reproductively senescent female mice (Zuloaga et al., 2014). 14,15-EET inhibited rat mesenteric arterial endothelial senescence, down-regulated p53 expression in aged endothelial cells and improved the impaired endothelium-dependent vasodilatation in aged rats through activation of mTORC2/Akt signaling pathway (Yang et al., 2014). Injection of 11,12-EET accelerated wound healing, tissue repair and reduced production of proinflammatory factors TNFα, IL-6, MMP and IL-1β in type 2 diabetic models of B6.VLepob/J (leptin-deficient, *ob/ob*) mice. In another study, both forms of EETs 11,12 and 14,15 epoxyeicosatrienoic acid rescued deteriorated wound healing in ischemic conditions (Sommer et al., 2019). Orally active subset of 14,15-EET analogs, termed EET-A, EET-B, and EET-C22 reduced cardiac and renal injury in spontaneous and angiotensin hypertension as well as promoted recovery after Ischemia/Reperfusion Injury by reducing contractile dysfunction (Campbell et al., 2017). Although several studies have highlighted the deleterious impact of cellular senescence in numerous chronic diseases, it is also recognized for its beneficial role in tumor suppression, wound healing and tissue regeneration. These studies highlight the contribution of CYP generated pro-resolvin lipid mediators in wound healing.

Oxidized Phospholipids

Polyunsaturated fatty acyl containing phospholipids are prone to lipid peroxidation and form oxidized phospholipids (OxPLs) that play a significant role in apoptosis and inflammation. OxPLs are generated enzymatically through lipoxygenases or non-enzymatic attack through reactive oxygen species. However, irrespective of the mode, identical primary oxidation products (i.e., peroxyl radicals and hydroperoxides) are produced initially that subsequently undergo further oxidation in a stochastic manner and release a heterogenous mixture of bioactive OxPL species. In general, lipid peroxidation and levels of OxPLs increase with age. OxPLs generated via cytoplasmic hydroxyl radicals exacerbated cellular senescence in Toll-like receptor 4-knockout (TLR4-/-) OKD48- and IDOL-Tg mouse models (Sakai et al., 2019). Inhibition of TLR2 with oxid ized-1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC), a well-studied oxidized product of a common phospholipid in mammalian cell membranes, 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) significantly reduced IL-1α and IL-1β mRNA induction in oncogene induced senescence of IMR90 cells. In another study targeting OxPLs by transgenic overexpressing of E06-svFv, a single chain variable fragment that binds to phosphatidyl choline head group of OxPL attenuated age-associated trabecular bone loss in both female and male mice (Palmieri et al., 2021). Similar E06-svFv transgene mechanisms to inactivate OxPLs have been studied with age-associated diseases such as atherosclerosis, steatohepatitis (Sun et al., 2020) and macular degeneration (Handa et al., 2015) suggesting neutralizing OxPLs as a promising therapeutic target for some of the age-associated diseases. Cardiolipins, major phospholipids in mitochondria are extremely prone to lipid oxidation. With mitochondria being the critical organelle handling oxidative stress, oxidation of cardiolipin can lead to organelle dysfunction and generate lipid peroxides further exacerbating oxidative stress and promoting senescence. Knock out of ALCAT-1, an enzyme that promotes cardiolipin peroxidation protected against onset of cellular senescence and preserved mitochondrial function by reducing sensitivity to lipid oxidation.

Greenberg et al. (2008) proposed the "lipid whisker" hypothesis as a novel feature of membrane architecture in senescent or apoptotic cells. These cells are distinguished by the presence of protruding "whiskers" as a result of phospholipid oxidation via lipid peroxidation or oxidative stress. The oxPL whiskers such as oxidized phosphatidylserine contribute to pattern recognition for common receptors like CD36 that play a major role in engulfment and phagocytosis of senescent cells (Podrez et al., 2002; Greenberg et al., 2006, 2008).

Sphingolipids

Sphingolipids are a class of complex phospholipids with a hydrophobic core consisting of an amino alcohol, sphingosine and a long-chain fatty acid chain. Bioactive forms this class such as sphingosine, sphingosine-1-phosphate, ceramide, ceramide-1-phosphate, dihydroceramide are known to modulate a plethora of cellular functions such as cell migration, growth regulation, adhesion, apoptosis, senescence and inflammatory responses (Hannun and Obeid, 2018).

Several studies have highlighted the role of sphingolipids in both development and aging (Giusto et al., 1992; Lightle et al., 2000; Cutler and Mattson, 2001; Hannun and Obeid, 2018). For example, aging induces accumulation of sphingosine and ceramides in liver and brain tissues and alterations in sphingolipid metabolism increases the risk and progression of age-related disease (Giusto et al., 1992; Lightle et al., 2000). A systemic study on sphingolipid metabolic enzymes in kidney, liver and brain tissues of day-1 to 720-day-old rats revealed a significant increase in activities of the sphingolipid catabolic enzymes (SMase and ceramidases) (Sacket et al., 2009). Impairment of ceramide and sphingolipid synthesis genes in

yeast, worms and flies extended lifespan (Cutler and Mattson, 2001; Rao et al., 2007; Yi et al., 2016).

In senescent human dermal fibroblasts (HDF), ceramide levels and a sphingomyelinase activity were markedly elevated. Exogenous ceramide induced senescent phenotype in young HDF at concentrations that mimics endogenous ceramide levels in senescent cells and recapitulated molecular changes of senescence, suggesting ceramides act as mediators of senescence (Venable et al., 1995). Of note, in therapy-induced senescence, exogenous addition of sphingomyelin increased ceramide levels that induced apoptosis and decreased senescence. This is possibly explained by ceramide levels- no inhibition of cell cycle progression at low ceramide levels, induction of senescence at moderate levels, and initiation of apoptosis at high levels (Modrak et al., 2009). In another study, deficiencies in ceramide transfer protein (CERT) in primary mouse embryonic fibroblasts led to senescence and dysregulated sphingolipid metabolism by increasing hexosylceramides production (Rao et al., 2007). Increased hexoceramides such as glucosylceramide led to age-related impairments in CD4(+) T cell function and immunosenescence (Molano et al., 2012). Several studies have suggested that sphingolipid metabolism as effectors or modulators of the p53 tumor suppressor pathway, particularly sphingomyelinase and sphingosine kinase I (SK1), which are directly regulated by p53, contributing to ceramide-induced senescence in cells (Dbaibo et al., 1998; Oskouian et al., 2006; Heffernan-Stroud and Obeid, 2011). For example, the expression of sphingosine kinase I (SK1), a downstream target of p53, was significantly elevated in p53-knockout mice that developed thymic lymphoma, and p53-deficient mice lacking SK1 were protected from thymic lymphoma. Mechanistic studies revealed that p53 tumor suppression caused by loss of SK1 was mediated by increased sphingosine and ceramides, resulting in tumor senescence. Non-canonical sphingolipids such as deoxyceramides has been shown to be depleted during tumor-induced senescence and increasing the levels of 1-deoxyceramides reduced the senescence phenotype in human colorectal cancer cells. The imbalance in deoxyceramide levels can affect sphingolipid signaling and alter membrane remodeling (Millner et al., 2020). Despite the fact that several evidences point to the multiple roles of bioactive sphingolipids in aging, these intriguing findings warrant more research into the functional role of sphingolipids in senescence.

Lysophospholipids

Lysophospholipids are multifunctional lipid mediators and are formed from hydrolyzed phospholipids with either an alkyl or acyl chain (Rivera and Chun, 2008). The lysophospholipids (LPLs) are broadly categorized as lysoglycerophospholipids and lysosphingolipids. The common ones in circulation are lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), lysophosphatidylserine (LPS), sphingosine, sphingosine-1 phosphate (S1P), and sphingosylphosphorylcholine (SPC) that are derived from corresponding phospholipids. Among the LPLs, lysophosphatidylcholine is the most abundant class (Ojala et al., 2007) and activates multiple signaling pathways that are involved in oxidative stress and inflammatory responses (Kakisaka et al., 2012; Bansal et al., 2016). They are also major secretory components of extracellular vesicles, which has recently gained a lot of attention as key players within the secretome of senescent cells (Urbanelli et al., 2016; Buratta et al., 2017). For example, biochemical characterization of extracellular vesicles released by fibroblasts subjected to H-Ras induced senescence indicated a significant enrichment in LPCs species (16:0; 16:1; 18:1; 18:2; 20:4, and 26:0). Both, replicative- and stress-induced-senescent dermal fibroblasts showed elevated levels of lysophosphatidylcholines. The LPC exhibited SASP activity by eliciting chemokine release in non-senescent fibroblasts and obstructed TLR2–6/CD36 signaling in macrophages (Narzt et al., 2021). Cholangiocyte senescence in biliary tract cancer has been linked to an increase in LPC levels and the resulting cytotoxicity. LPCs induced upregulation of components of SASP interleukin-8 (IL-8), IL-6, transforming growth factor-β and PAI-1, as well as p21 and SA-β-gal activity in MMNK-1 human cholangiocyte cells.

Studies have also shown association between aging and lysophosphatidic acid (LPA) signaling. For instance, alterations in LPA1 receptor was linked to the presence of depression and cognitive deficits in the elderly population (Moreno-Fernandez et al., 2018). Age-dependent changes in cAMP profiles were induced by LPA as noted in young and senescent human fibroblasts (Jang et al., 2006). LPA were detected at significantly higher concentrations in the cerebral cortex synaptosomes of aged rats (Pasquare et al., 2009). Knockdown of LPA3 led to cell senescence in mesenchymal stromal cells (Kanehira et al., 2017). Another finding showed that LPA regulates ROS levels and cell senescence through LPA3 to alleviate cell aging in Hutchinson–Gilford progeria syndrome (Chen et al., 2020). Together these studies reveal a key role of lysophospholipid signaling and its wide spread effects in the process of aging and senescence. **Figure 2** illustrates the key lipid players involved in senescence.

MAJOR MOLECULAR PLAYERS OF CELLULAR SENESCENCE AND THEIR ROLE IN LIPID REGULATION

p16

Cellular senescence is initiated and maintained through two critical pathways: $p16^{INK4A}$/Rb and/or p53/$p21^{cip1}$. It is important to note that both, p16 and p53, are well known tumor suppressors. p16 inhibits cyclin dependent kinases, CDK4 and CDK6, thus maintaining Rb in a hypophosphorylated form and blocking cell cycle progression (**Figure 3**). Expression of p16 is a key factor for maintaining long-term senescence (Serrano et al., 1997; Yamakoshi et al., 2009). A recent study isolated high expressing p16-positive cells, specifically macrophages, and showed that these cells exhibit several other markers of senescence, including SA-β-gal, reduced proliferation and accompanying SASP (Liu et al., 2019). This suggests that high expression of p16 is indeed associated with cellular senescence. Additionally, several single nucleotide polymorphisms (SNPs) have been identified in the *CDKN2a/b* locus (encoding the

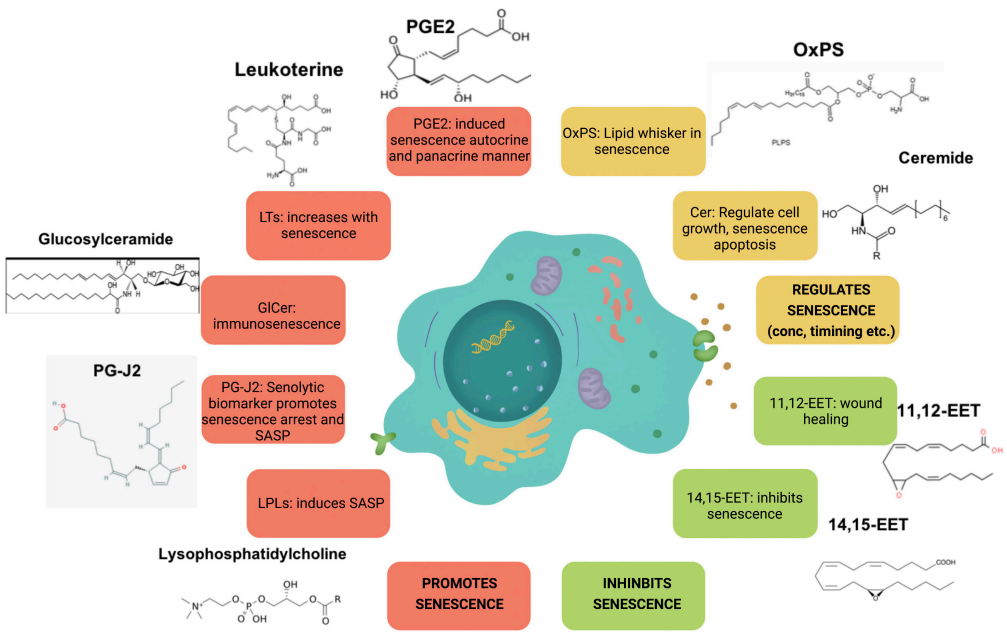

FIGURE 2 | Major lipid species involved in senescence. Species illustrated in red increase with senescence, whereas species in orange and green regulate and inhibit it, respectively.

p16INK4a, *ARF*, and *p15INK4b* transcripts) and associated with multiple age-related pathologies (Jeck et al., 2012; Fortney et al., 2015). Furthermore, p16 expression is largely undetectable in young mice, Krishnamurthy et al. (2004), Liu et al. (2009) but is strongly associated with aging in multiple mouse tissues (Burd et al., 2013). Similarly, other "drivers" that accelerate aging features, for example high fat diet, drives p16 expression through epigenetic modulation of its coding region (Zhang et al., 2018). Consistent with p16 being a major player in cellular senescence, genetic ablation of p16-positive cells, using the INK-ATTAC (Baker et al., 2011) and p16-3MR mice (Demaria et al., 2014), reduced senescent cell burden and improved healthspan, both in a progeria model, as well as naturally aged WT animals. Of note, preventing the accumulation of $p19^{ARF}$-positive cells, another tumor suppressor that regulates p53 stability did not delay or prevent age-related pathologies (Baker et al., 2008), suggesting that p16 is crucial for maintenance of senescence, at least in mice models.

In humans, peripheral blood T lymphocytes exhibit increased expression of $p16^{INK4a}$ with age, tobacco smoking and physical inactivity. Similarly, p16-positive senescent T cells increase with age in mice (Yousefzadeh et al., 2021). In addition to aging, senescent T cells have been isolated from a number of tumors including lung and breast cancer (Meloni et al., 2006; Gruber et al., 2008; Urbaniak-Kujda et al., 2009). These senescent T cells from the tumor microenvironment seem to be dysfunctional and have reduced antitumor activity (Liu et al., 2018; Zhao et al., 2020). Interestingly, such senescent T cells with increased p16 expression display unbalanced lipid metabolism, leading to accumulation of lipid droplets (Liu et al., 2021), consistent with senescent cells in other tissues. Inhibition of the group IVA phospholipase A_2, reprogrammed metabolism and prevented T cell senescence, suggesting that unbalanced lipid metabolism could be a key driver of T cell senescence (Liu et al., 2021). However, whether p16 directly controls lipid metabolism is still unknown in this model. One recent study did identify a direct link between p16 expression and lipid metabolism. During fasting, inhibition of p16 in primary hepatocytes enhanced activation of PPARα, a transcription factor with a major role in lipid metabolism, and increased fatty acid oxidation (Deleye et al., 2020; **Figure 3**). Consistent with this, p16 overexpression reduced mitochondrial activity and fatty acid oxidation, and led to accumulation of lipid droplets both *in vitro* and *in vivo*. However, p16 is known to have roles outside of senescence and these studies did not analyze cellular senescence in this fasting model. Of note, several studies (described above) suggest that elevated lipid β-oxidation is observed in senescent cells. Therefore, further studies exploring if p16 controls lipid catabolism during senescence will uncover insights into how p16 maintains long-term senescence.

p53

p53 is one of the most well-studied tumor suppressor, and is activated in response to various stressors including DNA damage, telomere attrition, mitochondrial, oxidative or oncogenic stress. Post-translational modification of p53, including phosphorylation, acetylation, sumoylation, ubiquitination and neddylation, regulate its activity. Upon acute stress, p53 is activated and can modulate DNA repair and quiescence (Vousden and Prives, 2009; Kasteri et al., 2018). However, chronic activation of p53 promotes cellular senescence (Sharpless and Depinho, 2006; Salama et al., 2014). p53 indirectly

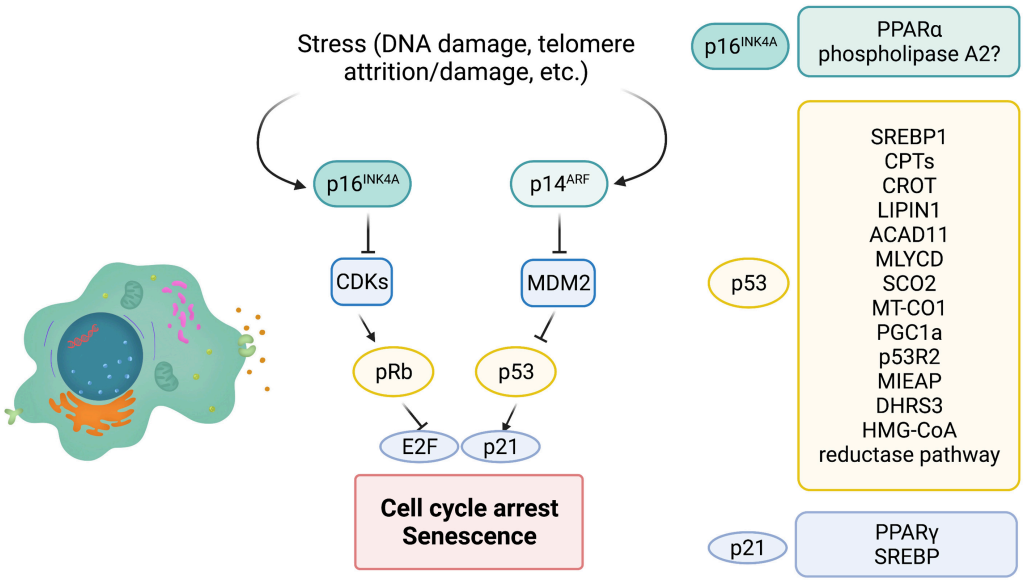

FIGURE 3 | The role of major players of senescence in lipid metabolism. P16^{INK4a}, p53, and p21 are major cell cycle regulators that control cellular senescence. INK4a/ARF encodes both p16 and ARF. MDM2 is an E3 ligase that ubiquitinates and leads to degradation of cellular senescence. ARF can inhibit MDM2 and therefore stabilize p53. P21 is a bonafide target of p53 and is partially responsible for cell cycle arrest and cellular senescence. P16 inhibits cyclin dependent kinases (CDK) thus maintaining Rb in a hypophosphorylated state and preventing E2F transcription factor activity. On the right are modulators of lipid metabolism that are regulated by p16, p53, and p21 (? = Possibly regulated by p16).

downregulates the expression of several genes required for cell cycle progression. Although the role of p53 in regulating the cell cycle is well-understood, recent studies indicate that p53 can also regulate metabolism and in particular lipid metabolism.

p53 enhances fatty acid oxidation and oxidative phosphorylation (OXPHOS), while inhibiting fatty acid synthesis. The role of p53 in suppressing lipid synthesis is prominent in the p53 knock out mouse models that exhibit obesity (Wang S. J. et al., 2016). Interestingly, induction of cellular senescence increases oxygen consumption and inhibits fatty acid synthesis, suggesting that p53 may play a pivotal role in rewiring the metabolic circuitry of senescent cells (Quijano et al., 2012). Inhibition of fatty acid synthesis by p53 is predominantly through repression of the master regulator of fatty acid synthesis, *SREBP1* (Yahagi et al., 2003). SREBPs control the expression of multiple lipogenic enzymes, including *acetyl-CoA carboxylase* (*ACC*), *ATP citrate lyase* (*ACLY*), and *fatty acid synthase* (*FASN*) (Yahagi et al., 2003; Eberle et al., 2004). These observations stem from a model of obese (*ob/ob)* mice, that displayed elevated *p53* and a concomitant downregulation of SREBP-1 and its targets, possibly by reducing promoter activity. Indeed, *p53* deletion in the *ob/ob* mice partially rescues SREBP-1 expression (Yahagi et al., 2003).

Carnitine palmitoyltransferase 1A and 1C (CPT1A, CPT1C), the rate limiting enzyme for fatty acid oxidation and aid in the transport of fatty acids into the mitochondria are transcriptionally regulated by p53 (Sanchez-Macedo et al., 2013). Carnitine *O*-octanoyltransferase (CROT) another enzyme that facilitates export of fatty acids from the peroxisomes has also been identified as a p53 target (Goldstein et al., 2012). Once exported from the peroxisomes these fatty acids can undergo mitochondrial β-oxidation. Similarly, Acad11 that catalyzes the first step of mitochondrial fatty acid oxidation, is a transcriptional target of p53 (Jiang et al., 2015). DNA damage with doxorubicin, an agent that induces double strand breaks and activates p53, upregulated Acad11 expression in a p53-dependent manner (He et al., 2011; Jiang et al., 2015). Malonyl-CoA decarboxylase (MLYCD) is a mitochondrial enzyme that converts malonyl-CoA to acetyl-CoA and a critical regulator of fatty acid oxidation. In a recent study in mice exhibiting ribosomal stress, MLYCD transcription was controlled through p53 binding to its promoter and intron regions, and reduced lipid accumulation in the liver (Liu et al., 2014). Another bonafide p53 target, Lipin1 has three p53 binding sites in intron 1, and is important for adipose tissue development. DNA damage, particularly double strand breaks (DSBs) by γ-irradiation, that activates p53 leads to increased Lipin1 expression in several tissues including spleen and thymus (Assaily et al., 2011). Recent data in mouse myoblast C2C12 cells, shows that upon glucose starvation p53 upregulates Lipin 1 and fatty acid oxidation (Assaily et al., 2011). Additionally, Lipin1 has also been shown to interact with PGC-1α, another p53 target, to promote fatty acid oxidation (Finck et al., 2006; Sen et al., 2011). Clearly, activated wild-type p53 does seem to play a role in lipid metabolism, particularly fatty acid oxidation. However, whether chronic p53 activation that is known to promote cellular senescence, leads to similar changes has not yet been fully elucidated. Fatty acid oxidation is linked to OXPHOS, and provides reduced equivalents to the OXPHOS pathway. p53 has several targets that play a role in OXPHOS, including *cytochrome c oxidase 2* (*SCO2)* (Matoba et al., 2006)

and the *mitochondrial encoded cytochrome c oxidase 1* (*MT-CO1*) (Okamura et al., 1999). p53 is also shown to translocate into the mitochondria and can interact with mitochondrial DNA polymerase γ (Achanta et al., 2005), control mitochondrial biogenesis through PGC1α, regulate mitochondrial DNA copy number and mitochondrial mass through *p53-controlled ribonucleotide reductase* (*p53R2*) (Bourdon et al., 2007) and is also involved in clearance of dysfunctional mitochondria by mitophagy through mitochondria-eating protein (Mieap) (Kitamura et al., 2011).

Interestingly, short-chain dehydrogenase reductase (DHRS3) that is localized to lipid droplets has also been identified as a target of p53 (Beilstein et al., 2016). There are two p53 responsible elements in the *DHRS3* promoter, and several reports show that activated p53 promotes buildup of lipid droplets concomitant with an increase in DHRS3 (Kirschner et al., 2010; Deisenroth et al., 2011). In addition to FAO, OXPHOS and lipid droplet dynamics, p53 is also involved in cholesterol homeostasis. Caveolin 1 is a structural protein that binds to cholesterol. In an *in vitro* study, in human dermal fibroblasts, *caveolin 1* was induced upon overexpression of p53 (Bist et al., 2000). This led to reduced intracellular cholesterol, suggesting the possible role of p53 in cholesterol efflux. The HMG-CoA reductase pathway is important for cholesterol synthesis and several enzymes in this pathway are p53 targets including 3′-hydroxy-3′-methylglutaryl-coenzyme A reductase, MVA kinase, farnesyl diphosphate synthase and farnesyl diphosphate farnesyl transferase 1 (Laezza et al., 2015). However, there are opposing reports on whether p53 activates or represses the HMG-CoA reductase pathway. Both studies use WT p53 expressing cell lines, however, one study uses astrocytes and glioblastoma cell lines whereas the other uses mouse embryonic fibroblasts, SK-HEP-1 and HCT116 cells (Laezza et al., 2015; Moon et al., 2019; **Figure 3**). Currently the role of HMG-CoA reductase pathway in cellular senescence is not clear, although a few reports suggest that inhibiting this pathway in pre-malignant cells promotes cellular senescence and inhibits tumorigenesis.

p21

A bonafide transcriptional target of p53 is $p21^{WAF1/CIP1}$, a 21 kDa protein encoded by the CDKN1A gene (el-Deiry et al., 1993). p21 interacts with cyclin dependent kinases (CDKs), thus maintaining RB in a hypophosphorylated state and inhibiting cell cycle progression (Harper et al., 1993; Chen et al., 1996). Since, p21 binds several cyclin/CDK complexes, including CDK1, 2, 4 and 6, it can promote arrest at any stage of the cell cycle. In contrast p16 specifically binds to inactivate CDK4 and CDK6, thus arresting cells in G0/G1 phase (Harper et al., 1993; Pavletich, 1999). p21 can also bind proliferating cell nuclear antigen (PCNA) (Waga et al., 1994) and inhibit the cell cycle. It is important to note that p21 can be activated in a p53-independent manner (Aliouat-Denis et al., 2005; Abbas and Dutta, 2009). As mentioned above, p16 expression is required to maintain a senescent state. In contrast, p21 is crucial for initiation of senescence (Noda et al., 1994; Hernandez-Segura et al., 2017). Indeed, mice lacking $p21^{WAF1/CIP1}$ showed defects in embryonic senescence leading to developmental defects (Munoz-Espin et al., 2013; Storer et al., 2013). Of note, embryonic senescence is a transient program thought to be beneficial and is resolved in a timely manner. Furthermore, several reports show that p21 expression does not persist in senescent cells (Stein et al., 1999; Sharpless and Sherr, 2015).

Role of p21 in lipid metabolism is not well understood. p21 does have an apparent role in adipocyte maintenance, however, outside of adipogenic effects not much is known about p21 in regulating lipid metabolism. There are some studies that report p21 as a pro-adipogenic factor. Knockdown or loss of p21 inhibited adipocyte differentiation (Inoue et al., 2008). Consistent with this, phosphorylation of p21 by MPK-38 led to nuclear translocation and increased association with peroxisome proliferator-activated receptor γ (PPARγ), thus inhibiting the role of PPARγ in adipogenesis. Furthermore, in this study expression of p21 rescued the metabolic phenotypes observed. Contradicting these studies, loss of p21 showed an increase in adipocyte hyperplasia (Naaz et al., 2004). In this study loss of p21 led to spontaneous adipose conversion and adipocyte hyperplasia in mice. However, effects in the study of Inoue et al. were observed in the terminal differentiation phase of the adipogenic program, whereas the anti-adipogenic effects were evident in the earlier determination phase of adipogenesis. Interestingly, p21 was identified as a target of SREBPs. Both SREBP-1a and SREBP-2 binding sites exist in the p21 promoter (Inoue et al., 2005). Furthermore, SREBP-1a led to p21 expression and cell cycle inhibition, under conditions of lipid deprivation. Consistent with this observation, SREBP null mice had reduced expression of p21 (**Figure 3**). It will be of great interest to examine whether expression of p21 leads to differential lipid changes during "acute" versus "chronic" senescence. Although, p16, p53, and p21 are key molecular players in cellular senescence their exact role in modulating lipid metabolism, reprogramming lipid composition and generation of bioactive lipid species is not well explored.

POTENTIAL ROLE OF LIPIDS AS THERAPEUTICS IN TARGETING SENESCENCE

Cellular senescence is a defining feature of aging, as well as a contributor to chronic non-healing wounds and general tissue aging pathology. Several key bioactive lipids such as cytochrome P450 (CYP)-derived epoxyeicosatrienoic acids (EETs), leukoterines, lipoxins described above are known to play a significant role in tissue repair. In fact, EETs have been shown as a viable angiogenic therapeutic strategy for its ability to accelerate wound epithelization (Sander et al., 2013). Long term inhibition of PGE2 degradation significantly reduced pulmonary fibrosis lesions in mouse model of bleomycin-induced lung senescence. In contrast, profibrotic leukotriene synthesis is elevated in IPF. Although PGE2 inhibition holds great promise as a stand-alone treatment or in combination with currently approved therapies for idiopathic pulmonary fibrosis, modulating eicosanoid metabolism in context of senescence can open up therapeutic avenues and provide mechanistic clues into tissue aging. Although the above studies have highlighted the

beneficial role of senescent cells in wound healing, it is still a paradox as to how these senescent cells also impair wound healing in aging. A temporal lipidome analysis of senescent cells from different tissues would provide insights into lipid changes that occur in senescent cells of aged tissues, as well as allow for the development of tailored senescence-targeting therapeutics.

TOOLS TO IDENTIFY FUNCTIONAL LIPIDS AND CURRENT CHALLENGES

The ubiquitous and structurally diverse nature of lipids molecules and its varied abundance across cells and tissues contributes to the complexity in the process of lipid identification. There are various techniques available for studying individual lipids and the lipidome, each with its own set of constraints. Understanding the benefits and limitations of these approaches is therefore crucial in identifying lipids within a biological model. With the emergence of lipidomics, there has been several advancements in analytical techniques such as soft ionization methods, high resolution mass spectrometry, tandem mass spectrometry, MS imaging and development of spectral libraries and lipid database such as LIPIDMAPS. This has led to not only identifying novel lipids but also unravel mechanisms responsible for pathophysiological conditions, biomarkers for early diagnosis and drug targeting (Quehenberger et al., 2010; Wang M. et al., 2016). Advances in high-resolution lipidomics platforms have made it possible to investigate lipidome changes associated with aging and age-associated diseases (Wong et al., 2019). For example, both targeted and untargeted lipidomics approaches provided insights into phospholipid remodeling in senescent cells, led to the identification of 15d-PGJ2 as a biomarker of senolysis and elevated ceramide C16:0 levels as prognostic marker for functional decline (Wennberg et al., 2018; Millner and Atilla-Gokcumen, 2020; Wiley et al., 2021). Despite advancements made in this field, the complexity in terms of chemistry, diversity, and abundances necessitates the continued pursuit of technical innovation. For example, identifying certain isomers of eicosanoids, a variety of sphingolipids, phospholipid intermediates are still challenging to be quantified accurately. Developing the informatics tools or libraries to interpret a multitude of lipid species and large sets of lipidomics data has been challenging. Nevertheless, a lot of progress has been made toward fine tuning of high-resolution mass spectrometry and precise identification of lipid structures. Multi-omic data integration approaches, dynamic lipidomics to asses turnover kinetics, single cell lipidomics has generated a lot of interest and holds promise in advancing the field forward.

UNANSWERED QUESTIONS IN THE FIELD

With advances in technology, we have unraveled that lipids do play a role in cellular senescence. However, we are left with several unanswered and interesting questions: Do different stages of cellular senescence (e.g., initiation versus late senescence) have different lipid compositions? If so is it causal in initiation or maintenance of senescence? While the lipids discussed in this review have been linked to senescence, more research is needed to establish cause or consequence effect. In addition to this, the link between major senescent players- p53, p21, or p16, and lipid alterations is still to be uncovered. Importantly, it will be interesting to understand whether lipids could act as biomarkers for senescence and/or senolysis, hence making intervention possible in the near future.

Cellular senescence, in summary is a cell-fate process associated with several age-related pathologies including frailty, Alzheimer's disease, Parkinson disease, atherosclerosis, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and osteoarthritis (Schafer et al., 2017; Chinta et al., 2018; Sagiv et al., 2018). Indeed, the National Institutes of Health (NIH) has recently started a new initiative, The Common Fund's Cellular Senescence Network (SenNet) Program, to better understand the key hallmarks of cellular senescence and map the burden of senescent cells in human. This further highlights the critical need of understanding cellular senescence. Additionally, directed removal of senescent cells or associated SASP improves health considerably, at least in animal models (Zhu et al., 2015; Jeon et al., 2017; Bussian et al., 2018; Guerrero et al., 2019; Zhang et al., 2019). Further understanding the role of lipids in cellular senescence will open new therapeutic approaches for several age-related diseases.

AUTHOR CONTRIBUTIONS

SH and AUG reviewed the literature, authored the review, contributed to the article, and approved the submitted version.

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REVIEW
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Acylation, a Conductor of Ghrelin Function in Brain Health and Disease

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Acyl-ghrelin (AG) is an orexigenic hormone that has a unique octanoyl modification on its third serine residue. It is often referred to as the "hunger hormone" due to its involvement in stimulating food intake and regulating energy homeostasis. The discovery of the enzyme ghrelin-O-acyltransferase (GOAT), which catalyses ghrelin acylation, provided further insights into the relevance of this lipidation process for the activation of the growth hormone secretagogue receptor (GHS-R) by acyl-ghrelin. Although acyl-ghrelin is predominantly linked with octanoic acid, a range of saturated fatty acids can also bind to ghrelin possibly leading to specific functions. Sources of ghrelin acylation include beta-oxidation of longer chain fatty acids, with contributions from fatty acid synthesis, the diet, and the microbiome. In addition, both acyl-ghrelin and unacyl-ghrelin (UAG) have feedback effects on lipid metabolism which in turn modulate their levels. Recently we showed that whilst acyl-ghrelin promotes adult hippocampal neurogenesis and enhances memory function, UAG inhibits these processes. As a result, we postulated that the circulating acyl-ghrelin:unacyl-ghrelin (AG:UAG) ratio might be an important regulator of neurogenesis and cognition. In this review, we discuss emerging evidence behind the relevance of ghrelin acylation in the context of brain physiology and pathology, as well as the current challenges of identifying the provenance of the acyl moiety.

Keywords: acylation, acyl-ghrelin, unacyl-ghrelin, octanoic acid, ghrelin-O-acyl transferase, neurogenesis, neurodegeneration, beta-oxidation

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INTRODUCTION

Ghrelin is a stomach peptide often referred to as "the hunger hormone" due to its ability to stimulate the sensation of hunger during periods of low energy balance (Date et al., 2000; Nakazato et al., 2001; Kojima et al., 2009; Tamboli et al., 2017).

Within the CNS, ghrelin regulates brain plasticity involved in regulating memory and anxiety (Hornsby et al., 2020). Although its complex physiological effects have just begun to be characterized, ghrelin is now considered a critical factor in gut-brain signaling with relevance to neurodegenerative diseases, particularly dementia (Buntwal et al., 2019).

The activity of ghrelin is dependent on a specific post-translational step, whereby the peptide is acylated, mainly by octanoic acid, on the third amino acid, a serine residue to form acyl-ghrelin (Kojima et al., 1999). In the absence of acylation (the attachment of the fatty acid) ghrelin cannot bind and activate GHS-R. In this review, we focus on the biochemistry of acyl-ghrelin function.

Ghrelin is a 28 amino acid peptide, first identified in 1999 as the endogenous ligand for the growth hormone secretagogue receptor-1a (GHS-R1a) and is essential for growth hormone (GH) release. Acyl-ghrelin mediates the beneficial effects associated with calorie restriction (CR) in neurodegenerative disorders, including Parkinson's Disease (PD) (Andrews et al., 2010). Indeed,

TABLE 1 | The amino acid sequence of ghrelin from a range of vertebrate species.

Mammalian	**1 ***	**10**	**20**	**28**
Human	GSSFLSPEHQRVQQRKESKKPPAKLQPR			
Mouse/Rat	GSSFLSPEHQKAQQRKESKKPPAKLQPR			
Dog	GSSFLSPEHQKLQQRKESKKPPAKLQPR			
Rhesus monkey	GSSFLSPEHQRAQQRKESKKPPAKLQPR			
Bovine	GSSFLSPEHQKLQ - RKEAKKPSGRLKPR			
Sheep	GSSFLSPEHQKLQ - RKEPKKPSGRLKPR			
Avian	1 *	10	20	26
Chicken	GSSFLSPTYKNIQQQKDTRKPTARLH			
Turkey	GSSFLSPAYKNIQQQKDTRKPTARLHPR			
Goose	GSSFLSPEFKKIQQQNDPAKATAKIH			
Fish	1 *	10	20	23
Rainbow trout	GSSFLSPSQKPQVROGKGK-PPRV-amide			
Goldfish	GTSFLSPAQKPQ—GRRPPRM-amide			
Zebrafish	GTSFLSPTQKPQ—GRRPPRV-amide			

For each species, the third amino acid of the ghrelin peptide sequence is a serine residue and is the location for acyl-modification (indicated by asterisks). As shown, there is homology between the NH_2-terminal sequences of ghrelin from these vertebrate species.

pre-clinical models demonstrate that acyl-ghrelin promotes neurogenesis and enhances memory function (Walker et al., 2015; Hornsby et al., 2016; Kent et al., 2015; Menzies et al., 2014; Moon et al., 2014), whilst we have recently shown that UAG reduces neurogenesis and impairs memory function, with clinical data suggesting that the circulating AG: UAG ratio may be a valuable diagnostic biomarker of dementia (Hornsby et al., 2020). Thus, as the acylated form of ghrelin is essential for the function and activation of GHS-R1a—acyl-ghrelin is often considered to be the 'active' form of ghrelin. Of note, the Ser3 ghrelin residue, along with its acylation, are both conserved in vertebrates (**Table 1**) (Kojima and Kangawa, 2005). Mutation of this serine residue prevents the acylation of ghrelin by ghrelin-o-acyltransferase (GOAT) (Yang et al., 2008b), unless the substitution is for threonine (Darling et al., 2015), which is consistent with threonine being present at this position in certain amphibians (Kaiya et al., 2001).

The acylation of ghrelin shares differences and similarities with other types of protein lipidation, the process through which a variety of lipids including cholesterol, isoprenoids and fatty acids are covalently attached to proteins during and/or after translation. These modifications including glycosylphosphatidylinositol (GPI) anchor, cholesterylation, N-myristoylation, palmitoylation, and prenylation regulate protein localization to membranes, protein interactions and function in signaling cascades. However, whilst lipidation was historically regarded as a relatively static modification providing membrane affinity or anchoring, the recent study of ghrelin and other proteins that are rapidly and reversibly acylated has rendered protein acylation a new signaling pattern relevant to many pathways. Although the acylation of ghrelin was the first demonstration of such a post-translational modification of a peptide, other important proteins such as the oncogenic regulator Wnt (Coombs et al., 2010; Herr and Basler, 2012; Janda et al., 2012; Willet et al., 2003; Nile and Hannoush, 2016; Takada et al., 2006), Hedgehog precursor proteins (Chamoun et al., 2001; Chen et al., 2004), and histocompatibility antigens (Schultz et al., 2018) can also be acylated by fatty acids that alter signaling capabilities. These alterations are modulated by the lipid actively participating in the mechanistic interactions that induce the conformational change required by the protein to exert its function. The linked lipid is therefore not a passive bystander but actively participates in the cascade of events. Interestingly, ghrelin, hedgehog and Wnt proteins are all acylated by members of the diverse family of membrane-bound O-acyl transferase (MBOAT) enzymes. Nonetheless, ghrelin is seemingly unique in its ability to bind fatty acids of varying lengths with higher specificity for octanoic acid. Studies into the physiological effects of post-translational acyl modifications are warranted.

The human ghrelin (GHRL) gene on chromosome 3p25-26 (**Figure 1**) encodes a 117-amino-acid precursor peptide, preproghrelin, which undergoes proteolytic cleavage in the endoplasmic reticulum (ER) to produce a 94 amino acid proghrelin peptide (Zhu et al., 2006). This is cleaved in the Golgi by pro-hormone convertase 1/3 (PC1/3) to generate the mature 28 amino acid ghrelin peptide (Liu et al., 2011). Notably, both proghrelin and ghrelin can be acylated by the enzyme GOAT (Zhu et al., 2006; Yang et al., 2008b).

In humans, intravenous acyl-ghrelin has a half-life of ~9–13 min (Liu et al., 2011). Indeed, ~90% of ghrelin detected in the circulation is UAG (Hosada et al., 2003), suggesting that its acylation is a rapid, reversible and tightly regulated process. Notably, within several cell types and tissues, ghrelin can be enzymatically acylated or de-acylated to permit fine cell-specific modulation of GHS-R1a signaling (Zeidman et al., 2009). Although several studies suggest that acyl-ghrelin and UAG may have distinct functions, with UAG no longer regarded as an inactive form of the peptide, it must be highlighted that for the rapid interconversion of UAG into acyl-ghrelin, basal levels of the peptide precursor are required, together with the necessary fatty acid.

ACYLATION OF GHRELIN BY GHRELIN-O-ACYLTRANSFERASE

In 2001, the human medullary thyroid carcinoma cell line (TT) was shown to generate UAG, enzymatically acylate UAG to become acyl-ghrelin, and secrete this into the media (Kanamoto et al., 2001). The subsequent generation of a ghrelin acylation cell culture system led to the identification of GOAT as the enzyme catalyst (Gutierrez et al., 2008). Also in 2008, mouse genome analysis identified membrane bound-O-acyl transferase (MBOAT4) as the gene (located on chromosome 8p12) that encodes for GOAT (Yang et al., 2008a). In agreement, acyl-ghrelin is not detected in GOAT null mice (Gutierrez et al., 2008) and the inhibition of GOAT leads to a decreased presence

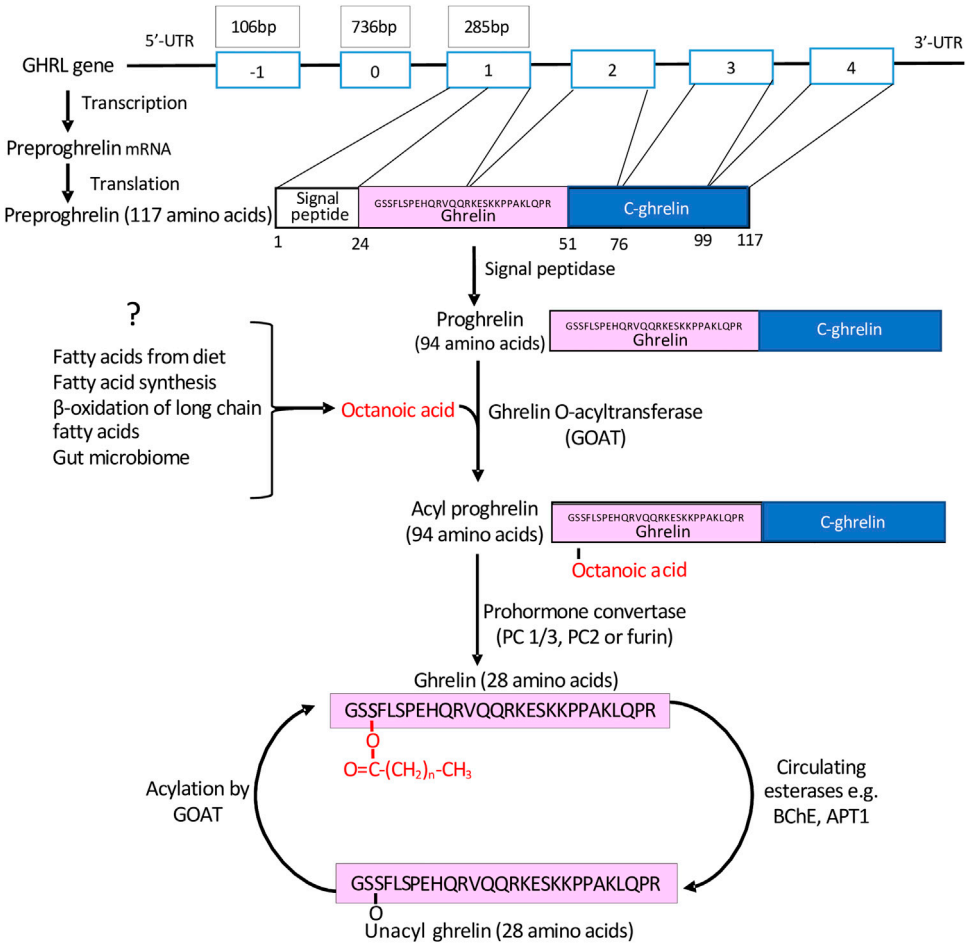

FIGURE 1 | The generation of mature acyl-ghrelin. Following transcription and translation of the GHRL gene, a preproghrelin peptide of 117 amino acids is generated. This undergoes proteolytic cleavage to generate proghrelin (94 amino acids). Either proghrelin or mature ghrelin (28 amino acids) can undergo acylation by GOAT, which is predominately bound by C8. Acyl-ghrelin may be deacylated by esterases and then reacylated by GOAT at an alternative location.

of acyl-ghrelin in insulinoma cells (Yang et al., 2008a), HEK and HeLa cells (Barnett et al., 2010) as well as in mouse serum (Yang et al., 2008a).

Other than GOAT, only two additional members of the MBOAT family, Porcupine (Porcn) and hedgehog acyl transferase (Hhat), are known to transfer fatty acids onto proteins. Other members transfer fatty acids to more complex lipids (Hofmann, 2000; Chang and Magee, 2009). GOAT is a membrane-bound enzyme-containing 11 transmembrane domains and is predominantly expressed in the stomach (Hofmann, 2000). The structure of GOAT is not fully resolved, and the enzyme's active site and substrate binding site are still unknown (Taylor et al., 2013). However, it contains two highly conserved residues: asparagine (Asn307)—which seems to be essential for the interactions with the substrates, and histidine (His338)—thought to be involved in the catalytic mechanism (Taylor et al., 2013; Masumoto et al., 2015). Knowledge of GOAT's ability to recognize ghrelin and fatty acyl-CoA substrates was obtained from biochemical and functional studies (Van Der Lely et al., 2004; Yang et al., 2008b; Darling et al., 2013; Darling et al., 2015) that suggest ghrelin as the unique substrate for GOAT (Darling et al., 2015). Generally, GOAT localization closely follows ghrelin expression, with high expression in the gastric oxyntic mucosa of rats and mice (Stengel et al., 2010), and humans (Gutierrez et al., 2008; Lim et al., 2011). However, GOAT is also expressed in the brain, particularly in the hippocampus (Murtuza and Isokawa, 2018) which is also an area of abundant GHSR-1a mRNA (Zigman et al., 2006; Hornsby et al., 2020) and protein expression (Mani et al., 2014; Hornsby et al., 2016). The central expression of GOAT and GHSR-1a suggest that local acylation and subsequent receptor activation following the import of UAG may be possible (Murtuza and Isokawa, 2018). Notably, GOAT mRNA is reduced in brain samples from AD patients (Gahete et al., 2011) and is specifically reduced in the granule cell layer within the dentate gyrus (DG) of PD Dementia (PDD) patients compared to both PD and healthy control groups (Hornsby et al., 2020). These studies suggest that acylation of ghrelin is associated with dementia in humans.

ACYL-GHRELIN BINDING TO GHS-R1A

Several experimental and *in-silico* strategies have been used to understand the rules for acyl-ghrelin binding and activating GHS-R1a. Liposome binding, zeta potential and isothermal calorimetry were used to show that acyl-ghrelin—but not UAG—penetrated the headgroup and partially into the lipid backbone regions of membranes (Staes et al., 2010). Importantly, acylation increased the concentration of the peptide in the membrane 120-fold, increasing its chances of engaging its receptor. Of note, several hydrophobic amino acids at the N-terminal—where the octanoyl moiety is attached—were shown to be critical for the close association of ghrelin with membranes. Consistent with this, a model of membrane-associated ghrelin inferred from Rosetta modelling based on NMR studies of the peptide in lipid vesicles suggest that ghrelin binds to membranes *via* the octanoyl moiety on Ser3 and with the contribution of basic amino acids electrostatically interacting with the lipid polar heads (Vortmeier et al., 2015). These amino acids were later characterized and the N-terminal binding motif of acyl-ghrelin was confirmed to substantially contribute to the binding energy of the peptide-receptor complex and ghrelin proposed to assume a rigid helical conformation involving C-terminal residues (Bender et al., 2019). This two-site binding mode where the octanoyl chain stabilizes the structure of the hormone and promotes its binding to the receptor was later confirmed (Ferré et al., 2019). Here, the utilization of solid-state NMR of lipid nanodiscs combined with coarse-grained molecular dynamic simulations substantially confirmed previous models (Bender et al., 2019) and experimental data (Bednarket et al., 2000; Matsumoto et al., 2001). The octanoyl chain strongly facilitates access of acyl-ghrelin to deeper binding pockets of the receptor as compared to UAG. Subsequently, acyl-ghrelin binds the receptor establishing a tight interaction with N-terminal residues, leading to the specific formation of a hydrophobic core, while the C-terminal region remains flexible. The hydrophobic core gives rise to the most stable peptide-receptor interactions which rigidifies the peptide in the bound state. However, the acyl chain seems not to interact with specific amino acids engaging in specific receptor-ligand interactions but rather effectively contributing to the formation of the hydrophobic core required to engage the deeper binding sites (Ferré et al., 2019). These data agree with previous studies showing that substitution of the C8 moiety with bulky hydrophobic amino acids are tolerated while polar or charged modifications hamper binding and activation of the receptor. As discussed, the preference for the octanoyl fatty acid likely resides in GOAT's acyl-chain specificity (Darling et al., 2015). The crystal structure of the receptor bound to a neutral antagonist was resolved and in combination with mutagenesis and receptor binding and activity analyses, results showed that GHS-R1a shared similarities with both peptide hormone GPCRs and lipid GPCRs while possessing unique structural features. Particularly, the binding pocket of the receptor is uniquely crossed by a salt bridge—with mutations completely abolishing function—dividing it into two cavities. Mutations of polar amino acids in cavity 1 with hydrophobic alanine also abolish activity demonstrating their involvement in peptide recognition. However, similarly to lipid GPCRs, the ghrelin receptor also presents a wide gap (crevasse) which is enriched with hydrophobic phenylalanine residues, spacing between trans-membrane bundles 6 and 7. In the prototypical lipid GPCRs, cannabinoid-1 and sphingosine-1-phosphate receptors, similar phenylalanine residues recognize the lipoligands, whilst the ghrelin receptor accommodates the acyl moiety of ghrelin into this hydrophobic environment (**Figure 2A**) (Shiimura et al., 2020). These interactions allow for acyl-ghrelin recognition and are postulated to induce the transformation of the receptor into its active form according to a mechanism common to class A GPCRs (Zhou et al., 2019). Adding further complexity, cryo-electron microscopy structures of the ghrelin receptor in complex with an engineered G-protein and bound either to acyl-ghrelin or to a synthetic agonist show a bifurcated cavity with the N-terminus of acyl-ghrelin deeply inserted and occupying almost the entire binding pocket (Wang et al., 2021). Again, mutations of the amino acids involved in the salt bridge and of the hydrophobic amino acids in the binding pocket largely decreased acyl-ghrelin's activity—supporting the critical role of these structures in binding and activating the receptor. However, different from previous modelling studies, here the octanoyl group is located at cavity 2 instead of being inserted in a crevasse near cavity 1. Additionally, the authors also propose that after binding in cavity 2, the octanoyl group orients the rigid N-terminus towards cavity 1 leading to initiation of signal transduction through conformational changes involving specific transmembrane bundles 6 and 7. Finally, several micro-switches or small conformational changes are induced to facilitate receptor-G protein coupling. The authors also postulate that hydrophobic interactions at the bundles above, together with the permanent salt bridge may be involved in basal activity, as this decreases severely upon mutation of the amino acids involved (**Figure 2B**) (Wang et al., 2021).

IN SITU BRAIN ACYLATION OF GHRELIN AND CROSSING THE BLOOD-BRAIN BARRIER

The brain is shielded by the blood-brain barrier (BBB) which selectively restricts access to the brain. Both acyl-ghrelin and UAG can be imported directly across the BBB from the periphery, with the acylation status reported to alter its ability to cross the BBB into the parenchyma (**Figure 3**) (Banks et al., 2002). Banks et al. demonstrated that radio-labelled human acyl-ghrelin crossed the mouse BBB *via* a saturable brain import and export system, whilst mouse acyl-ghrelin was only saturable during brain-to-blood transport. As only two of the 28 residues differ between mouse and human ghrelin these particular amino acids may be critical for recognition and transport from the blood to the brain, but not necessarily for brain-to-blood transport. The same study also reported that mouse UAG accessed the brain *via* non-saturable transmembrane diffusion, suggesting that it might move from the circulation into the brain for local acylation by GOAT

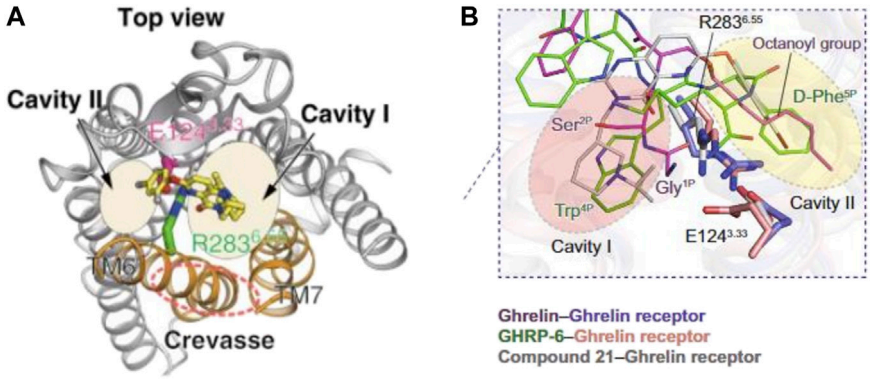

FIGURE 2 | Structure modelling of the ghrelin receptor whilst bound to ghrelin. Structures proposed by Shiimura et al. (2020) **(A)** and Wang et al (2021) **(B)** both identify the ligand-binding pocket of ghrelin receptor being bifurcated into two cavities. The model proposed in **(A)** presents the octanoyl group inserted in a crevasse near cavity 1. Whilst the model proposed in **(B)** locates the octanoyl group within the second cavity.

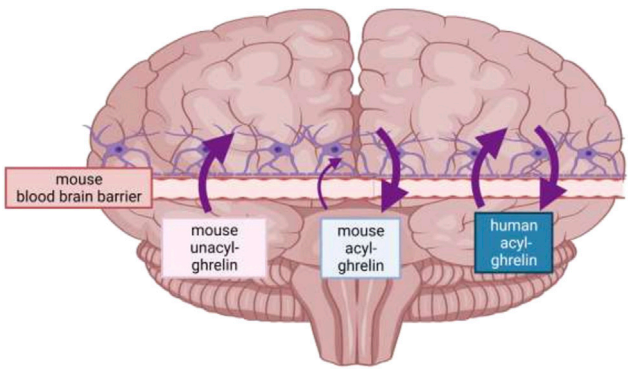

FIGURE 3 | Direction of travel of human acyl-ghrelin, mouse acyl-ghrelin and mouse unacyl-ghrelin across the mouse BBB. Mouse acyl-ghrelin predominantly crosses the mouse BBB in the brain-to-blood direction, mouse unacyl-ghrelin is transported only in a blood-to-brain direction, whilst human acyl-ghrelin, which differs from mouse ghrelin at two amino acid residues is transported in both directions across the mouse BBB. As a result, ghrelin transport across the BBB is influenced by at least two features of its structure-its amino acid sequence and post-translational acylation (created with Biorender, adapted from Banks et al., 2002).

(Murtuza and Isokawa, 2018). These initial findings were later supported by radiolabelled ghrelin studies suggesting that human acyl-ghrelin crosses the BBB *via* endocytosis (Diano et al., 2006; Pan et al., 2006). More recently, peripheral injections of F-ghrelin (a fluorescent analogue of acyl-ghrelin) resulted in its internalization by ependymal cells of the choroid plexus and by a specific subset of tanycytes, highly specialized ependymal cells that form a blood-cerebrospinal fluid (CSF) barrier (Uriarte et al., 2019). Whilst the exact mechanism of ghrelin's entry into the brain is unknown, acyl-ghrelin's accessibility seems to occur in a dose-dependent manner. Indeed, a low dose of a fluorescent acyl-ghrelin analogue injected subcutaneously in mice was scarcely present in the CSF and demonstrated a higher preference for the hypothalamus. While intracerebroventricular (icv) injected acyl-ghrelin (or high doses injected peripherally) was detected at high levels in the CSF and several brain regions (Cabral et al., 2013; Uriarte et al., 2019).

FATTY ACID SUBSTRATES FOR GHRELIN ACYLATION

In humans and rats, acyl-ghrelin is predominantly bound by octanoic acid (C8:0, a saturated eight carbon fatty acid, also commonly known as Caprylic acid) (Kojima et al., 1999). Although this appears to be the physiologically relevant form, ghrelin may bind a range of saturated fatty acids that vary in length from 2 to 16 carbons (Hosoda et al., 2003; Nishi et al., 2005b; Gutierrez et al., 2008). The development of a cell-based system whereby fatty acids added exogenously to cells resulted in the formation of acyl-ghrelin with the corresponding fatty acid chain used to demonstrate that even non-endogenous fatty acids can be employed for the bio-acylation of ghrelin (Gutierrez et al., 2008; Gutierrez et al., 2008; Oiso et al., 2013). However, given that ghrelin acylation may be modulated by increased bioavailability of specific fatty acids the discussion regarding the preferred acyl donor group for ghrelin acylation by GOAT resides in the enzyme specificity. Indeed, several studies report that C8:0 is the preferred fatty acid substrate for acylation by GOAT, with its affinity being approximately 50-fold higher in comparison to C6:0 (hexanoic acid) and C10:0 (decanoic acid) (Kaiya et al., 2001; Darling et al., 2015) and 10:1 (decanoic acid, a monounsaturated fatty acid) (Hosoda et al., 2003). Conversely, Ohgusu et al. (2009) reported that GOAT prefers C6:0 as the acyl donor, followed by C8:0 then C10:0, and may not attach longer fatty acids such as C16:0 (palmitic acid) to ghrelin. Yang et al. (2008b) also suggest that GOAT may not be able to incorporate palmitate onto ghrelin. Notably, the attachment of C16:0 (palmitoylation) or C14:0 (myristic acid, myristylation) are the most common forms of acylation for a wide range of proteins (for a review please see Resh, 2016).

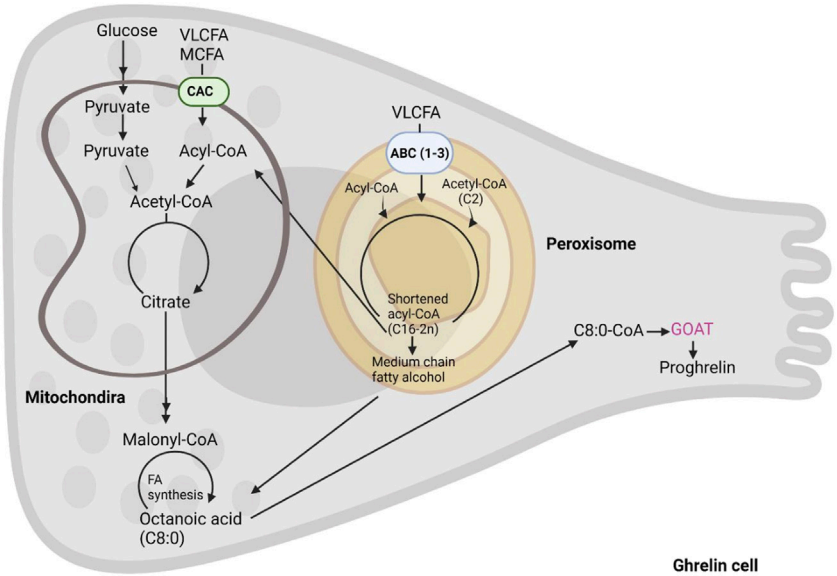

FIGURE 4 | The formation of octanoic acid in the enteroendocrine cell. Acyl-ghrelin is predominantly bound by C8:0. In this schematic we summarize the intracellular C8:0 forming pathways including, fatty acids from diet, fatty acid synthesis and beta-oxidation of long chain fatty acids.

Distinct species of acyl-ghrelin differ in their efficiency to activate GHS-R1a (Bednarek et al., 2000; Date et al., 2000; Kaiya et al., 2001; Matsumoto et al., 2001; Hosoda et al., 2003; Nishi et al., 2005a; Gutierrez et al., 2008; Heppner et al., 2012). A fatty acid chain greater than two carbons was needed for activating GHS-R1a (Heppner et al., 2012). In addition, C2:0 (acetic acid), C8:0, C12:0 (dodecanoic acid), C14:0 and C16:0 forms of acyl-ghrelin had distinct effects on food intake and fat mass. Interestingly, C16:0-ghrelin led to a delayed (~24 h) effect on stimulating feeding in comparison to the more immediate effect of C8:0-ghrelin (Heppner et al., 2012). Thus, studies to distinguish the acyl group added to ghrelin under both physiological and pathophysiological conditions are warranted. As part of this, one should consider the source of the acyl substrate and their respective metabolic pathways as targets to alter the profile of ghrelin species and subsequent GHS-R1a signalling.

There are several possible sources of fatty acids for the acylation of ghrelin, including, breakdown of longer-chain fatty acids (beta-oxidation), *de novo* fatty acid synthesis, and diet. Despite this the origin of the acyl moiety within the brain and stomach is still unknown. In addition, these processes may be modulated by the body's microbiome. Here, we discuss putative biochemical mechanisms that may underpin both basal and appetite-associated acylation of ghrelin in the circulation.

BETA OXIDATION OF LONGER CHAIN FATTY ACIDS

Fatty acids are a major source of energy in animals and the main pathway for their biological degradation, namely β-oxidation, is present in both mitochondria (Kennedyt and Lehninger, 1949) and peroxisomes (Lazarow and De Duve, 1976). The two organelles regulate this pathway with both similarities and differences due to their enzymatic inventory. For this review, "very long-chain" is defined as having a number of carbons >20, "long-chain" comprised between 20 and 16, "medium-chain" between 14 and 8, and "short-chain" < 8. β-oxidation is the process where fatty acids are degraded in a stepwise manner to support energy production. As relevant to this review, MCFA such as octanoic acid used for ghrelin acylation, as well as SCFA, are water-soluble and may enter cells and organelles through unselective membrane pores (Papamandjaris et al., 1998; Antonenkov and Hiltunen, 2012).

Conversely, hydrophobic LCFA and VLCFA utilize membrane transporters after being activated by esterification into acyl-CoA (**Figure 4**). The latter reaction is carried out by ACSL and FATP proteins whose differential localization within the two organelles is yet to be fully deciphered, for a review see Schrader et al. (2015). Also, the fatty acid transporters are different in mitochondria and peroxisomes where the former are equipped with the carnitine shuttle (CPT2, CACT, and CPT1) and the latter with three ABC transporters (ABCD1-3 (**Figure 4**)). Peroxisomes also possess a carnitine shuttle but whilst the mitochondrial transporter is used to import LCFA, the peroxisomal transporter exports MCFA. Indeed, after import, peroxisomes do not degrade fatty acids to completion, and according to the substrate affinities of their respective enzymes only shorten the chain length of fatty acids to approximately C8-C6. Intriguingly, C8-C6 are those preferentially used by GOAT for ghrelin acylation. These are exported from peroxisomes through a carnitine shuttle or free diffusion and then imported into mitochondria for further oxidation. In contrast to the mitochondrial carnitine shuttle, peroxisomal ABCD transporters prefer VLCFA (and dicarboxylic acids), thus the

initial transporters function as filters trafficking fatty acids to the two organelles depending on their chain length. These findings demonstrate that both mitochondrial and peroxisomal beta-oxidation may play a role in supporting ghrelin acylation by providing the necessary fatty acyl moieties. However, studies where ghrelin levels have been measured with concomitant inhibition or depletion of specific mitochondrial and peroxisomal beta-oxidation enzymes is warranted.

Both acylated and unacylated ghrelin can stimulate fatty acid oxidation in skeletal muscle (Kraft et al., 2019) and myocytes (Han et al., 2015), and continuous administration of acyl-ghrelin enhanced muscle mitochondrial oxidative capacity in rats (Barazzoni et al., 2005; Guillory et al., 2017). Bolus ICV injections of ghrelin to rats led to an increase in long-chain acylcarnitines, which is indicative of increased beta-oxidation (Gao et al., 2013). Acyl-ghrelin administration increased the activity of carnitine palmitoyl transferase (CPT1), which transports fatty acyl-CoA into mitochondria for oxidation in the mouse hypothalamus (Andrews et al., 2008). Furthermore, in a transgenic mouse model where genes for both human ghrelin and GOAT are overexpressed, there was significantly decreased mRNA expression of Cytochrome C and uncoupling protein-3 (UCP-3) in the skeletal muscle (Kirchner et al., 2009). These results are indicative of increased beta-oxidation in the mitochondria (Schrauwen et al., 2001; Andrews et al., 2005; Andrews et al., 2009; Andrews et al., 2010).

Several studies have investigated whether fatty acids longer than C8:0, particularly those introduced *via* the diet, can increase acyl-ghrelin. ST-Onge et al. (2014) showed that both MCT and LCT increased levels of acyl-ghrelin in overweight humans (St-Onge et al., 2014). Whilst intravenous infusion of LCFA increased both acyl-ghrelin and the AG: TG ratio in rats (Barazzoni et al., 2017). Could essential omega 3 fatty acids represent precursors for acylating ghrelin? Several studies link these particular fatty acids and ghrelin. Indeed, a fatty seafood diet, rich in DHA and EPA, increased ghrelin in the blood of humans (Ramel et al., 2009). Furthermore, omega-3 supplementation elevated serum ghrelin levels in both rats (Mostafa et al., 2020) and humans (Rahimi and Khademi, 2019). High-capacity runner rats fed a balanced diet (containing ×10 more fish oil, and therefore more omega 3 fatty acids) had higher plasma levels of acylated ghrelin in comparison to those on an unbalanced diet (Ramel et al., 2009). Furthermore, ghrelin levels in rat plasma were significantly higher after a high-fat high-sucrose diet supplemented with both DHA and EPA (1:1) and grape seed extract (Ramos-Romero et al., 2016). Consistent with these studies, LCFA was needed for the acylation of ghrelin in a ghrelinoma cell line (MGN3-1) (Bando et al., 2016).

Conversely, the oxidation of longer fatty acids may not be required for ghrelin acylation. For example, a 12-week dietary intervention where humans ingested either commercial goat cheese or goat cheese enriched with omega-3 PUFAs and linoleic acid did not lead to altered ghrelin levels (Santurion et al., 2020). Another study in humans compared low and high omega-3 dietary regimes for 6 weeks also showed no difference in serum ghrelin (Parra et al., 2008). More specifically, oleic acid (18:1) increased preproghrelin mRNA expression when cultured with intestines and hepatopancreas cells from goldfish (Bertucci et al., 2017). However, no effect was seen in response to linoleic acid (18:2) or EPA, whilst DHA led to a decrease in preproghrelin mRNA and protein (Bertucci et al., 2017). Furthermore, Haynes et al. (2020) reported that effects induced by octanoic acid on pro-opiomelanocortin (POMC) neurones, such as ion channel opening, were not subsequently seen by the LCFAs oleic and palmitic acids (Haynes et al., 2020). It seems that rather than being potential precursors for the acyl modification of ghrelin, some LCFA or VLCFA act to decrease acyl-ghrelin. Indeed, gastric gavage of LCFAs significantly decreased ghrelin secretion in the serum of mice (Lu et al., 2012). Also, the culture of ghrelin expressing gastric cells with docosadienoic acid (C22:2), linoleic acid (18:2) and palmitic acid (16:0) reduced ghrelin secretion (Lu et al., 2012). Oiso et al. (2015) investigated both the *in vitro* and an *in vivo* effect of different fatty acids (acetic acid (C2:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α-linoleic acid (18:3)) on octanoylated ghrelin in AGS-GHRL8 cells (a gastric carcinoma cell line that expresses ghrelin) (Oiso et al., 2015). They reported that stearic, oleic, linoleic and α-linoleic acids significantly reduced the amount of octanoyl-ghrelin secreted into the culture medium. Similarly, the administration of ethyl oleate (which can be converted to oleic acid in the body) to mice significantly reduced both octanoylated- and unacyl-ghrelin in serum (Oiso et al., 2015).

The ghrelinergic cell line (MGN3-1) α-linoleic acid led to a concentration-dependent decrease in octanoyl-ghrelin that seemed to be modulated by gustducin (Janssen et al., 2012). Conversely, the clearance of ghrelin may be mediated by the fatty acid receptor, GPR120 (Widmayer et al., 2017). A GPR120 agonist GW9508 significantly decreased the secretion of acyl-ghrelin, which was rescued after treatment with GPR120 siRNA. A fasting-induced increase in plasma acyl-ghrelin was also blocked in GW9508 treated mice (Gong et al., 2014).

In summary, several studies indicate that food consumption acts as an endogenous source of MCFA, including octanoic acid, for ghrelin acylation. However, it should be noted that in these studies the levels of acyl-ghrelin are the result of an equilibrium between acylation by GOAT and de-esterification by ATP1 or similar esterases. At present, and to the best of our knowledge, the effect on acyl-ghrelin following the use of fatty acid β-oxidation inhibitors has not been delineated in animals. However, should their findings be confirmed *in vivo* they may imply that beta-oxidation is the major source of octanoic for ghrelin acylation, considering that the same study also explored the potential impact of the intestinal microbiome and fatty acid synthesis onto the production of MCFAs for ghrelin acylation but did not find data in support. Fatty acids synthesis, the diet and the microbiome can, however, modulate ghrelin acylation and in a specific setting and are therefore reviewed in the following. In summarizing the findings above we may propose a feedback mechanism linking ghrelin and beta-oxidation. Inhibition of β-oxidation (with mitochondrial CPT1 inhibitors) decreased ghrelin acylation *in vitro* and many studies above have shown

that such inhibition also decreased its orexigenic effects. Therefore, beta-oxidation would be required both for ghrelin acylation and to mediate its effects (or at least some of them). Ghrelin also interacts with PPAR-γ which induces peroxisomal and mitochondrial biogenesis while interactions with PPAR-α or -δ, which can induce expression of beta-oxidation enzymes, are not known. These data suggest that ghrelin is stimulating β-oxidation (or at least preparing cells for that purpose) when substrate is low (e.g., during starvation) to prevent hypoglycaemia and death. Given that more data is needed to conclude beta-oxidation donating the C8 for acylation, we speculate that during periods of fasting/hunger beta-oxidation of adipose tissue sustains ghrelin acylation and in turn acyl-ghrelin positively upregulates beta-oxidation.

FATTY ACIDS FROM THE DIET

Analyses of acyl-ghrelin and medium-chain fatty acids (MCFAs) in human plasma during fasting report no correlation, suggesting that fatty acids for ghrelin acylation may preferentially derive from gastric content rather than plasma (Nass et al., 2015). The diet is a rich source of MCFA, with dairy products such as milk and oils from palm kernel and coconuts enriched in MCFAs such as octanoic acid (Jensen et al., 1990; Takeuchi et al., 2008). Ingested MCFA and medium-chain triacylglycerols (MCTs) (a glycerol backbone attached to three fatty acids) elevated levels of acyl-ghrelin within the stomach without inducing the expression of total ghrelin mRNA or peptide (Nishi et al., 2005b). More specifically, ingestion of either MCTs including glyceryl trihexanoate (3xC6:0), glyceryl tripheptanoate (3xC7:0), glyceryl trioctanoate (3xC8:0) or glyceryl tridecanoate (3xC10:0) or the corresponding MCFAs (hexanoic, octanoic or decanoic acids) by mice, increased the acyl-ghrelin species in the stomach containing the corresponding number of carbons i.e., hexanoyl-ghrelin, heptanoyl-ghrelin, octanoyl-ghrelin or decanoyl-ghrelin, respectively. However, this effect was not seen in mice fed the glyceryl tributyrate (C4:0), glyceryl trilaurate (C12:0) or glyceryl tripalmitate (C16:0) suggesting that fatty acids from C6 to C8 are preferred by GOAT. A subsequent study in 2005, showed that rats fed with chow containing glyceryl trioctanoate led to the detection of octanoyl-ghrelin in their gastric content (Nishi et al., 2005a). Mice fed with a MCT diet of triheptanoate, trioctanoate or tridecanoate led directly to increased levels of heptanoyl, octanoyl- or decanoyl-ghrelin, respectively, in the plasma (Kirchner et al., 2009). This study also included transgenic mice which overexpress both the human ghrelin (GHRL) and GOAT (MBOAT4) genes in the liver. These mice did not produce octanoylated human ghrelin when fed regular chow (only octanoylated mouse ghrelin), however, feeding with trioctanoate led to the presence of circulating octanoylated human ghrelin (Kirchner et al., 2009). Several other studies provide further support for diets enriched with octanoic acid increasing plasma acyl-ghrelin levels, including in plasma of pigs (Miller et al., 2016) and cachectic patients undergoing enteral feeding (Ashitani et al., 2009), demonstrating that acyl-ghrelin levels can be modulated by feeding. The same effect was reported in the stomachs of neonatal chickens (Yamato et al., 2005) and plasma, liver and stomach of suckling rats (Lemarié et al., 2016). Furthermore, a diet enriched with MCTs increased levels of acyl-ghrelin in overweight humans where acyl-ghrelin levels are low (Yamato et al., 2005). Fukumori et al. (2013) also showed that dairy cows fed a diet with increased content of C8:0, C10:0 and C12:0 led to increased plasma total ghrelin (TG) (Fukumori et al., 2013). Of particular interest, a diet enriched with octanoate did not lead to an increase in plasma acyl-ghrelin in rats, but rather led to a decrease in UAG, resulting in an increased AG:TG ratio. Acyl-ghrelin detected in gastric cells correlated with the dose of dietary tioctanoin, which supports the theory of direct absorption of the fatty acyl substrate for ghrelin acylation *via* the gut (Lemarié et al., 2015).

Conversely, Kaur et al. (2020) showed that the addition of glyceryl trioctanoate to the diet of pregnant mice increased total ghrelin but not acyl-ghrelin (Kaur et al., 2020). However, this study did not compare the levels of acyl-ghrelin detected in mice on glyceryl trioctanoate to mice on standard chow, but rather, to mice on a diet enriched with glyceryl tripalmitate (C16:0). Their rationale being that dietary palmitate does not lead to ghrelin acylation (Nishi et al., 2005b). However, as C16:0 can bind ghrelin (Gutierrez et al., 2008) or even be a precursor for octanoic acid (*via* beta-oxidation), these results should be interpreted carefully. Most of the studies described above suggest that dietary-derived MCFAs or MCTs can be used for ghrelin acylation in the gut.

Indeed, GOAT may act as a gastric lipid sensor, linking ingested nutrients with the hypothalamic energy balance regulation *via* the ghrelin system. More specifically, the activation of GOAT in the gut is suggested to link to the availability of the lipid pool, or more specifically to the MCFA content, as MBOAT4 mRNA expression is downregulated in the absence of MCFA (Kirchner et al., 2009). However, a diet with increased glyceryl octanoate did not affect the mRNA expression of ghrelin or MBOAT4 in pregnant mice (Kaur et al., 2020). There is also evidence that UAG may promote the uptake of fatty acids by cells for acylation. Indeed, using a fluorescent form of C12:0, UAG increased the uptake of this fatty acid into cardiomyocytes (Lear et al., 2010). In summary, dietary MCT may directly influence the ghrelin species present in the stomach, however, further investigation of other tissues is required in this context.

FATTY ACID SYNTHESIS

During dietary insufficiency, the brain (Knobloch et al., 2013), liver (Dorn et al., 2010) and adipose tissues (Mayas et al., 2010) can synthesize fatty acids (Skulachev 1991; Menendez and Lupu 2007; Knobloch et al., 2013). During this process, fatty acid synthase (FAS) can synthesize longer chain fatty acids (Maier et al., 2006) (**Figure 4**). However, MCFA synthesis has only been reported in goat mammary glands (Knudsen and Grunnet, 1982). More specifically, the synthesis of octanoic acid has been described in the lactating glands of rats (Libertini and Smiths,

1978) and octanoylated ghrelin has also been detected in the lactating mammary glands of both dairy goats (Zhang et al., 2018) and humans (Grönberg et al., 2010). However, several studies suggest that the fatty acid for ghrelin modification is not predominantly derived from fatty acid synthesis. For example, Lopez et al. (2008) reported that fasting and ghrelin treatment led to a decrease in FAS in the rat hypothalamus, which is regulated by the metabolic sensor, AMPK. Acyl-ghrelin treatment of rats with a bolus intracerebroventricular (ICV) injection led to a decrease in Malonyl-CoA and long-chain acyl-CoA in their ventral medial nucleus, which are indicative of decreased fatty acid synthesis (Gao et al., 2013). Furthermore, neither acylated nor UAG altered the incorporation of fatty acids into TAGs or phospholipids (Kraft et al., 2019). AMPK-mediated phosphorylation of acetyl-CoA carboxylase (ACC)-1 inhibits fatty acid synthesis (Lopez et al., 2008; Peterson et al., 2012; Adams, 2004), whilst phosphorylating ACC-2 promotes beta-oxidation (O'Neill et al., 2014; Fullerton et al., 2013). Notably, mRNA expression of the SCFA receptor, GPR43, was higher in gastric-derived ghrelin positive cells in comparison to ghrelin negative cells. However, incubation of these with butyric acid (C4:0) or valeric acid (C5:0) did not affect ghrelin secretion (Lu et al., 2012). The SCFA, acetate (C2:0), reduced appetite after crossing the BBB (Frost et al., 2014), suggesting that it could act as a competitive inhibitor of octanoyl-ghrelin. Further work is required to determine whether fatty acid synthesis and ghrelin acylation are directly linked, particularly in the context of cell-type specific ghrelin action.

FATTY ACIDS FROM THE MICROBIOME

The co-habitant bacteria in the gut, or the microbiome, is also a source of fatty acids that could be used to acylate ghrelin. The most abundant gut microbiota-derived metabolites include the SCFAs acetate (C2:0), propionate (C3:0) and butyrate (C4:0) (Topping and Clifton, 2001), which are synthesized during the fermentation process of nutrients in the colon. There are also reports of the microbiome utilizing diet-derived MCFAs and LCFAs (Zhao et al., 2018; Radka et al., 2020). For example, MCFAs and LCFAs have been shown to promote and inhibit different types of pathogenic or symbiotic bacteria, respectively, in addition to having antibacterial properties (Fischer et al., 2012; van der Hoeven-Hangoor et al., 2013). The gut microbiome has been associated with ghrelin function. Generally, the microbiome plays a role in numerous activities which overlap with the function of GHSR-1a, including obesity (Torres-Fuentes et al., 2017), adult hippocampal neurogenesis (Ogbonnaya et al., 2015) and metabolism (Cox et al., 2014). More directly, acetate derived from gut microbiota can stimulate ghrelin secretion (Perry et al., 2016), whilst circulating levels of ghrelin are seemingly linked to the microbiome. For example, ghrelin levels were negatively correlated with *Lactobacillus, Bifidobacterium*, and *B. coccoides Eubacterium rectale*, but positively correlated with *Bacteroides* and *Prevotella* (Queipo-Ortuño et al., 2013). Interestingly, germ-free mice have decreased ghrelin in their blood (Duca et al., 2012). Also, a probiotic diet in chickens led to an increase in ghrelin gene expression in their proventriculus (the glandular section of their digestive system) (Poorghasemi et al., 2018). Also, the culture of primary stomach cells from mice with *Lactobacillus brevis* SBC8803 resulted in an increase in acyl-ghrelin in the supernatant (Saito et al., 2019). Importantly, the ratio of AG: UAG was also significantly increased in rats 30 min after oral administration of *Lactobacillus brevis* SBC8803 (Saito et al., 2019). Notably, the transcription of ghrelin or GOAT mRNA was unchanged, suggesting that the increase may be due to promoting the acylation of ghrelin. Furthermore, bacteria-derived SCFAs (Cherbut et al., 1997; Dass et al., 2007) and LCFAs (Zhao et al., 2018) have been linked to increased gut motility, a function of acyl-ghrelin signaling (Fujimiya et al., 2012). However, a recent report suggested that the microbiome did not modulate plasma acyl-ghrelin in mice (Ikenoya et al, 2018). Whether the gut microbiome can regulate brain function *via* the acylation of ghrelin has yet to be fully investigated.

FATTY ACIDS INTO THE BRAIN

The ability of fatty acids to traverse the BBB or the blood-CSF barrier should be considered. Tight junctions formed in the BBB mean that free fatty acids enter the brain either *via* passive diffusion or they are assisted by proteins, however, the exact mechanism for fatty acid import is still unclear. Fatty acid diffusion depends on the length of the fatty acid chain which can impact their solubility (Kamp et al., 2003; For a review see Hamilton, 1998). SCFAs and MCFA's traverse the BBB by diffusion whilst transport of longer chain fatty acids is slower and requires assistance, for example, by the fatty acid transport protein (FATP)-1 (Kamp et al., 2003). Both essential fatty acids, such as linoleic acid (18:2) (DeMar et al., 2006) and non-essential fatty acids e.g., arachidonic acid (20:4) and palmitic acid (16:0) are derived from the blood (DeMar et al., 2006). Importantly, octanoic acid enters the brain *via* diffusion, shown *in vitro* (human U373-MG GBM cells (Yamazaki et al., 1996), in brain slice preparations (Kuge et al., 2002) and in rats (Yamazaki et al., 1996), dogs (Kuge et al., 2000) and cats (Kuge et al., 2000), where the ability of radiolabelled octanoic acid to enter the brain was quantified using brain PET imaging. Furthermore, octanoic acid administered directly into the CSF of mice was rapidly transported into the hypothalamus *via* GPR40 (Haynes et al., 2020). Radiolabelled octanoic acid administered into the mouse gut by oral gavage also entered the hypothalamus, whilst an oral gavage of the MCT octanoate decreased food intake and increased melanocortin secretion from the hypothalamus, a hormone that is linked to regulation of energy homeostasis (Haynes et al., 2020). In the same study, octanoic acid readily crossed the BBB whilst LCFA (oleic and palmitic acids) transport was minimal. This is supported by radiolabelled dietary palmitic (16:0) or oleic acid (18:1) fed to rat pups remaining undetected in the brain, whilst other polyunsaturated fatty acids such as linoleic acid were detected in the brain (Edmond et al., 1998; Williams et al., 1997). However, C14 labelled oleic acid was reported to transport across confluent cultures of primary human brain microvessel endothelial cells (HBMEC) (Mitchell et al., 2009).

Notably, essential fatty acids such as DHA and EPA cannot be synthesized by the body and must be derived from the diet. Thus, the fatty acid for ghrelin acylation in the brain may either be derived directly from the periphery or longer chain fatty acids that may be selectively imported across the BBB.

Collectively, the studies discussed in this review increase our understanding of ghrelin acylation and the underlying biochemistry. Further work unpicking the cell-specific mechanisms governing the acylation of ghrelin will likely yield novel insights into the integration of energy balance with brain plasticity linked to hippocampal neurogenesis and cognition.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

ACC Acetyl-CoA carboxylase

AD Alzheimer's disease

AEBSF 4-(2-Aminoethyl)benzensulfonyl fluoride hydrochloride

AG Acyl-ghrelin

AHN adult hippocampal neurogenesis

APT Acyl-protein thioesterase

BBB blood brain barrier

BChE butyrylcholinesterase

BDNF brain derived neurotrophic factor

CNS central nervous system

CR calorie restriction

DG dentate gyrus

DHA docosahexaenoic acid

EPA eicosapentaenoic acid

ER endoplasmic reticulum

FAS fatty acid synthase

FATP fatty acid transport protein

FFA free fatty acid

FFARs free fatty acid receptors

GHRL ghrelin

GHS-R1a growth hormone secretagogue receptor-1a

MBOAT4 membrane bound O-acyl transferase

GOAT ghrelin-o-acyltransferase

GPCR G-protein coupled receptor

Hhat hedgehog acyl transferase

LC-MS liquid chromatography-mass spectrometry

LCT long-chain triglycerides

MAFP Methoxy arachidonyl fluprophosphate

MALDI-TOF Matrix-Assisted Laser Desorption/Ionization-Time of Flight

MCFA medium chain fatty acids

MCT medium chain triacylglycerols

MS mass spectrometry

NSPC neural stem and progenitor cells

PAF platelet activating factor

PC1/3 pro-hormone convertase 1/3

PD Parkinson disease

PDD Parkinson disease dementia

PUFA polyunsaturated fatty acid

SCFA short-chain fatty acid

SGZ sub granular zone

UAG unacyl-ghrelin

UCP uncoupling protein

VLCFA very long-chain fatty acid

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