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Metabolic Labeling of Glycerophospholipids via Clickable Analogs Derivatized at the Lipid Headgroup

Christelle F. Ancajas, Tanei J. Ricks, Michael D. Best

Department of Chemistry, University of Tennessee, 1420 Circle Drive, Knoxville, TN, 37996 (USA)

Abstract

Metabolic labeling, in which substrate analogs containing diminutive tags can infiltrate biosynthetic pathways and generate labeled products in cells, has led to dramatic advancements in the means by which complex biomolecules can be detected and biological processes can be elucidated. Within this realm, metabolic labeling of lipid products, particularly in a manner that is headgroup-specific, brings about a number of technical challenges including the complexity of lipid metabolic pathways as well as the simplicity of biosynthetic precursors to headgroup functionality. As such, only a handful of strategies for metabolic labeling of lipids have thus far been reported. However, these approaches provide enticing examples of how strategic modifications to substrate structures, particularly by introducing clickable moieties, can enable the hijacking of lipid biosynthesis. Furthermore, early work in this field has led to an explosion in diverse applications by which these techniques have been exploited to answer key biological questions or detect and track various lipid-containing biological entities. In this article, we review these efforts and emphasize recent advancements in the development and application of lipid metabolic labeling strategies.

Keywords

metabolic labeling; Phospholipids; lipids; Synthesis; Phosphatidylcholine; Phosphatidic Acid; Phosphatidylinositol

1. Introduction

The ability to selectively label and analyze target biomolecules within their native environments of living cells and organisms has long been a prominent goal of biological research (Prescher and Bertozzi, 2005). This endeavor has faced profound challenges due to the vast complexity of metabolic networks, in which a plethora of variations of each biomolecule are constantly undergoing nuanced transformations that alter structure and thereby dictate function, which are often dysregulated in disease (Cairns et al., 2011; Santos and Schulze, 2012). Therefore, it is exceedingly difficult to focus in on one particular

mdbest@utk.edu.

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biomolecule or family of compounds for study. Despite these challenges, the potential payoff for this pursuit is very high, as these techniques enable the tracking of key features including biosynthesis, subcellular localization, and trafficking that are at the core of many fundamental biological questions (Best, 2009; Devaraj, 2018; Sletten and Bertozzi, 2009, 2011). Within this realm, lipid metabolic labeling strategies, which employ synthetic substrate analogs bearing diminutive click chemistry tags that are capable of hijacking metabolic pathways and producing tagged products in cells, have emerged as invaluable chemical tools (Scheme 1) (Bumpus and Baskin, 2018). In this arena, strategies for the effective labeling of important lipid targets have begun to be reported, which have paved the way for initial applications of this strategy to address biological questions. These recent advancements provide the subject for this review article.

A key challenge that needs to be overcome to achieve the production of labeled versions of products in cells pertains to issues associated with modifying natural substrates, and particularly that these modifications may suppress entry into metabolic networks. For this reason, many early labeling strategies were fairly conservative, primarily focusing on isotopically labeled versions of native substrates, which have the benefit of most closely resembling native substrates due to the subtle introduction of heavy atoms. As a result, experiments exploiting the production of isotopically labeled lipid products have been invaluable for understanding a variety of aspects of lipid metabolism and activity (Ecker and Liebisch, 2014; Wegener et al., 2016). Nevertheless, particularly in early studies, this approach has been limited in terms of the ability to image the localization of lipid products, although that has now changed due to advancements in mass spectrometry-based imaging methods (Berry et al., 2011; Bowman et al., 2019; Murphy et al., 2009). Cellular fluorescence microscopy is a stalwart technique in interrogating biological systems due to the ability to analyze changes in molecular subcellular localization (Bolte and Cordelieres, 2006). However, this requires the introduction of bulky fluorescent reporters onto labeled products, which for quite some time was not feasible for metabolic labeling strategies due to the dramatic alterations in structure this would necessitate for substrate analogs.

A key advancement in metabolic labeling came with the discovery of biorthogonal strategies for bioconjugation, involving reactions that utilize functional groups that are orthogonal to those that exist in biological systems (Best, 2009; Devaraj, 2018; Sletten and Bertozzi, 2009, 2011). Phrased another way, these reactions exploit probes bearing pairs of reactive tags that do not exist in biological systems (or minimally exist), do not react with other functional groups present in biology (or react as little as possible), but reliably react with one another under ambient conditions to produce stable labeled products. This was a longstanding challenge since, due to the vast complexity of functional groups present in nature, it is quite challenging to discover biorthogonal reactions. A breakthrough came through advancement of the ‘click chemistry’ philosophy, which focused on the development of high-yielding reactions that occur under ambient conditions utilizing ‘spring-loaded’ reactants (Kolb et al., 2001). These attributes translated into strong biorthogonal features for prototypical bioconjugation reactions including the copper catalyzed azide-alkyne cycloaddition (CuAAC) (Hein and Fokin, 2010; Meldal and Tornøe, 2008; Rostovtsev et al., 2002; Tornøe et al., 2002), and the Staudinger ligation between azide and phosphine reagents (Kohn and Breinbauer, 2004; Nilsson et al., 2000; Saxon et al., 2000; van Berkel et al., 2011) (Scheme

1). Please note that while numerous conditions and reagents have been developed for these reactions, we only show generalized reactions in Scheme 1. In addition, while we show specific combinations of probe tags and reagents in these figures, these can often be swapped (i.e. utilizing alkyne-tagged probes and azide-functionalized reagents).

An additional benefit of these biorthogonal conjugation reactions is that many of them employ reactive tags that are very small, such as azide and alkyne, consisting of only three and two atoms, respectively, for the tags themselves. This attribute was key for overcoming the challenge of developing suitable modified substrate analogs. Therefore, minimally modified substrate derivatives strategically altered by the addition of reactive tags such as azide and alkyne could act as effective precursors for biosynthetic machinery when fed to cells so as to produce labeled downstream products via native metabolic pathways. These products bearing clickable tags can then be post-modified with bulky reporter groups such as fluorescent dyes and biotin to enable selective interrogation of the progeny of the target probe. This transformative metabolic labeling procedure was first demonstrated for the labeling of cell surface glycoproteins using azide-modified mannose analogs as precursors for sialic acid-containing glycans (Saxon and Bertozzi, 2000). Since that time, metabolic labeling strategies have been extended to all families of biomolecules, including carbohydrates (Chang et al., 2009; Laughlin and Bertozzi, 2007; Tiwari et al., 2016), nucleotides (El-Sagheer and Brown, 2010; Gierlich et al., 2006; Gramlich et al., 2008; Seo et al., 2003), proteins (Johnson et al., 2010; Kiick et al., 2002; Liu and Schultz, 2010; Ngo and Tirrell, 2011; Wang et al., 2001), and lipids (Bumpus and Baskin, 2018; Jao et al., 2015a; Jao et al., 2009), and have led to dramatic advancements in the ability to scrutinize biological systems.

Concomitantly, numerous improvements have been made to biorthogonal reactions to balance reactant stability with reaction kinetics. A key advancement involved the development of the strain-promoted azide-alkyne cycloaddition (SPAAC) (Agard et al., 2004; Baskin et al., 2007; Jewett and Bertozzi, 2010), which circumvents the need for problematic copper catalyst in click chemistry reactions. In addition, new tags and reactions such as the inverse electron-demand Diels-Alder (IEDDA) cycloaddition reaction between tetrazine and *trans*-cyclooctene tags (Oliveira et al., 2017; Selvaraj and Fox, 2013; Wu and Devaraj, 2016) have expanded the toolbox for bioconjugation (Scheme 1).

Of the biomolecule families that have been studied, the development of metabolic labeling strategies targeting glycerophospholipids has progressed at a rather sluggish pace. However, these lipids represent a key target group for studies since they act as signaling molecules that regulate critical biological processes, their sub-cellular localization is a key aspect for controlling their function, and due to the vast complexity of lipid metabolic networks consisting of numerous combinations of lipid headgroups and acyl chain compositions that are constantly undergoing enzymatically driven interconversions. These attributes will be briefly introduced herein, and are conveniently covered in great detail in this special issue on lipid signaling.

Many lipids are now known to regulate key biological processes, for which aberrant lipid production correlates with high-profile diseases such as cancer (Bruntz et al., 2014; Griner

and Kazanietz, 2007; Katso et al., 2001; Mazhab-Jafari et al., 2015). These signaling roles of lipids are often complex and difficult to study, such as protein-lipid binding interactions that anchor proteins onto the membrane surface and thereby regulate function (Cho and Stahelin, 2005; Lemmon, 2008). Localization is also critical since different lipid species are known to be selectively localized within the membranes of distinct organelles. Furthermore, lipids self-assemble into domains such as lipid rafts, a phenomenon that also elicits key ramifications for function (Lingwood and Simons, 2010; Sezgin et al., 2017). These phenomena result from complex lipid biosynthetic machinery as well as lipid trafficking pathways that remain mysterious. For all of these reasons, lipids are important targets for metabolic labeling since they control key biological processes, they exhibit complex biosynthetic and localization patterns that are linked to their function and the onset of disease, and due to the inherent complexity of biological membranes.

2. Strategies for Metabolic Labeling of Glycerophospholipid Headgroups

Metabolic labeling strategies have built upon prior work in imaging lipids in cells. For example Schultz and co-workers demonstrated the ability to deliver and image clickable PA compounds in cells (Kuerschner and Thiele, 2014; Laguerre and Schultz, 2018). From there, early success was found in lipid metabolic labeling by utilizing fatty acid (FA) analogs bearing clickable tags including azide and alkyne at the terminus of the hydrophobic acyl chain. This approach circumvents the issue of obstructing biosynthetic transformations since these tags can be conveniently hidden at the end of these long hydrocarbon chains. As a result, clickable FAs have provided tremendously important probes for tracking fatty acid metabolism (Robichaud et al., 2016; Thiele et al., 2012) and characterizing proteins that undergo posttranslational lipidation processes by using strategies including post-derivatization via click chemistry, affinity chromatography purification, and mass spectrometry (MS)-based proteomics to identify labeled proteins (Hang and Linder, 2011; Hang et al., 2011). However, many applications of lipid labeling hinge upon the selective labeling of lipid molecules containing a particular headgroup. This is challenging to accomplish using FA probes, although Thiele and co-workers have recently reported a sophisticated multiplexed MS platform for tracking lipid metabolism (Thiele et al., 2019). Alternatively, modified substrate analogs have been developed that alter the lipid head group in order to differentiate between lipid molecules. In this endeavor, it is challenging to develop clickable precursors that can successfully mimic the native substrate for incorporation into specific lipid products. This aspect of lipid metabolic labeling will be the primary focus of this review article.

Before discussing recent labeling advances, we should take a quick peak at lipid metabolic networks, for which we will focus on a simplified picture of transformations in mammalian systems as shown in Scheme 2. As is typical of metabolic networks, here we find a complex series of interconversions highlighted by two major pathways that are linked by feedback loops. The Kennedy Pathway commences with choline and culminates in phosphatidylcholine (PC) (Gibellini and Smith, 2010), while the CDP-DAG pathway converts glycerol 3-phosphate through the intermediate phosphatidic acid (PA) to cytidine diphosphate diacylglycerol (CDP-DAG) (Athenstaedt and Daum, 1999). However, these pathways are linked via the hub of PC, PA, and diacylglycerol (DAG), which are

interconverted by phospholipase, phosphatase, and kinase enzymes, which have been key targets of metabolic labeling (Bumpus and Baskin, 2018). These central lipids exhibit significant differences; for example PC is the most abundant phospholipid in mammalian cells while PA and DAG are signaling lipids that are tightly maintained at low concentrations (Merida et al., 2008; Wang et al., 2006). These core pathways feed into the biosynthesis of downstream lipids including phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylserine (PS). PI acts as the precursor for the phosphatidylinositol polyphosphate (PIP_n) family (Best et al., 2010) as well as glycosylphosphatidylinositol (GPI) anchors (Orlean and Menon, 2007), the latter of which serve to anchor proteins onto the surfaces of cellular membranes. These complex metabolic networks are important to keep in mind when designing substrate analogs to target particular lipid species in order to scrutinize the different pathways by which labeled molecules will be formed and altered. Finally, it should be noted that the pathways shown in Scheme 2 are simplified and only depict transformations in mammalian cells, as lipid metabolic networks are often significantly different in varying organisms.

2.1. Labeling of PC and PA Products

A pioneering effort in this field was the report of the labeling of PC by Salic and co-workers in 2009 (Jao et al., 2009). To achieve this, the native substrate choline was modified into propargylcholine (**PCho**) to incorporate a clickable tag into PC products (Figure 1). This modification entails the conversion of one native methyl group into the new propargyl moiety, in essence adding two additional carbons by changing one hydrogen into a CCH unit, culminating in a minor manipulation to minimize potential steric clash in the active sites of biosynthetic enzymes. Previous studies had indicated that a methyl of the choline group could be replaced with an alkyl group of up to 5 carbons to produce metabolically active substrate analogs. Metabolic PC labeling via click chemistry was confirmed to be successful through dose-dependent intensity increases in fluorescence microscopy images of NIH 3T3 cells after treatment with a clickable partner azide-tagged fluorophore. This fluorescence was abrogated by treatment with phospholipase C (PLC) that decomposes PC, but restored upon treatment with PLC and EDTA, the latter of which occupies calcium that is needed for PLC activity. Fluorescence and immuno microscopy imaging experiments also showed that labeled PC molecules matched the subcellular localization of native PC, with products primarily localized at the Golgi and ER (site of biosynthesis) as well as the plasma membrane (PM) and mitochondria (to which PC is trafficked). Additionally, tandem MS experiments were performed to investigate incorporation, and observed mass peaks showed labeled PC products resulting from **PCho** for which acyl chain compositions closely mirrored those of native PC. Kinetic studies indicated that staining was readily observable within 30 minutes and intensity increased up to 24 hours, with pulse chase studies indicating that the labeled PC population remained stable. Finally, imaging studies were successful in mice. This result provided an exciting initial effort in lipid metabolic labeling. Since this pioneering report, **PCho** has also been shown to label myelin, as was developed in work aimed at studying remyelination (Aharoni et al., 2016), as well as teichoic acid glycopolymers attached to the peptidoglycan of *Streptococcus pneumoniae* (Di Guilmi et al., 2017). **PCho** has also been used to probe phosphorylcholine-derivatized proteins (Snodgrass

et al., 2015) and acts as an inhibitor of choline catabolism in bacteria (Fitzsimmons et al., 2011).

In subsequent work, a two-color labeling protocol was developed for imaging different pools of PC products in cells (Jao et al., 2015b). To do so, azido-choline substrate analogs were analyzed, and while both azidoethyl- and azidopropyl-choline were found to be effective, the former (**AECho**, Figure 1) was determined to be incorporated more efficiently. Impressively, almost 20% of PC lipids were found to contain an azidoethyl tag at the highest concentration and after 24 hours, even in the presence of 30 μ M of natural choline. For 2-color labeling, cells treated with either **PCho**, **AECho** or both were first labeled via SPAAC to selectively derivatize and image products labeled by **AECho** that contain an appended azide moiety. Subsequently, products resulting from **PCho** were labeled by CuAAC. While these two probes showed similar localization in proof-of-concept studies, this approach opens the door to differentiating pools of PC products generated under different conditions. Duan and co-workers also utilized **PCho** to develop a ratiometric labeling strategy for MCF-7 cells employing an azido-cresyl violet reagent that is blue-shifted upon CuAAC (Fu et al., 2015).

While the prior effort achieved infiltration of the Kennedy pathway, Baskin and co-workers have explored the labeling of lipid products of PC hydrolysis by phospholipase D (PLD) enzymes, which are important due to their regulation of processes such as cell growth, division, and migration, with aberrant behavior leading to disease. Their initial reports took advantage of the discovery that hydrolysis of PC by PLD, which normally produces PA, can be intercepted using short chain alcohols to produce phosphodiester analogs of PA. Initially, alkynols including 6-hexynol (**Hexynol**) were identified as substrates for PLD, but this required post-derivatization via CuAAC, which can be problematic due to copper toxicity (Bumpus and Baskin, 2016). Therefore, in follow-up work, 3-azidobutanol (**Azprop**) was explored as a precursor that is amenable to SPAAC derivatization. This approach was coined IMPACT (Imaging Phospholipase D Activity with Clickable Alcohols via Transphosphatidylation) (Bumpus and Baskin, 2017). Systematic studies including fluorophore labeling and analysis via fluorescence-coupled HPLC, MS analysis of labeled products after clicking on an ammonium group to enhance ionization, and cellular fluorescence microscopy imaging revealed robust labeling of PLD products in HeLa cells. These studies took advantage of the ability to stimulate PLD activity using the small molecule phorbol 12-myristate 13-acetate (PMA) and suppress activity using the PLD inhibitor 5-fluoro-2-indolyl deschlorohalopemide (FIPI) as well as isoform-selective inhibitors. Fluorescence imaging studies in conjunction with colocalization markers also confirmed that labeled products primarily appeared in the ER (attributed to isoform-selective inhibitors to PLD1) and Golgi apparatus (arising from a mixture of PLD1 and 2). The former result was somewhat surprising, as PA synthesis in the ER has primarily been thought to result from acylation of glycerol 3-phosphate (Scheme 2). Fluorescence recovery after photobleaching (FRAP) studies indicated rapid diffusion of labeled lipids in the ER but slower diffusion in the Golgi. Finally, enhanced IMPACT labeling in a subset of puncta led to the discovery that an mCHERRY-tagged PLD1 exhibited similar localization. Overall, these initial reports proved IMPACT to be a powerful tool for characterizing PLD activity.

Baskin and co-workers have recently advanced this work in different directions. First, IMPACT has been modified to develop a real-time imaging protocol for imaging PLD activity (Liang et al., 2019). To do so, it was required to circumvent wash protocols that are typically necessary to remove unreacted fluorophores after click chemistry postderivatization protocols. This was achieved by discovering that probe **(S)-oxoTCO-C1** bearing a bulky *trans*-5-oxocene (oxoTCO) unit was a suitable substrate for PLD enzymes that could be modified by an IEDDA reactions using a fluorogenic tetrazine reagent. Therefore, the fluorescent signal is dramatically enhanced (1,600-fold) following postlabeling, enabling real-time imaging by circumventing the need to remove unreacted dyes. This approach extended imaging of PA production beyond the ER and Golgi apparatus to enable visualization of labeled products at the plasma membrane (PM), which was attributed to the rapid time-scale of labeling. Furthermore, time-lapse movies captured trafficking of products out of the PM and into the ER and then the Golgi apparatus. Additional studies led to the conclusion that activation of PLD through the platelet-derived growth factor receptor (PDGFR) primarily impacted intracellular pools of PLD1, in contrast to activation by PMA or M1 muscarinic receptor (M1R), which acts primarily at the PM.

Additionally, the lab asked the question of whether propargylcholine probe **PCho** could also serve as a substrate for PLD enzymes (Bumpus et al., 2018). This effort was inspired by the potential of interrogating both PC and PA biosynthesis using a single probe. When HeLa cells were activated with PMA and treated with **PCho**, labeled PA products were again detected, primarily as a result of PLD1 activity based on isoform-selective inhibitor studies. This was achieved by identifying varying conditions for targeting each pathway; short (20 min) treatment and PMA stimulation to target PA labeling via PLD as opposed to long (24 h) incubation periods in the absence of PMA to produce tagged PC products.

2.1. Labeling of Inositol-containing Lipid Products

Beyond these core lipids, efforts have also recently emerged to label downstream lipid products. Much of this work has focused on *myo*-inositol-containing lipids due to the important signaling roles of these molecules. In pioneering work, Guo and co-workers pursued the labeling of glyphosphatidylinositol (GPI) anchors, which cause the membrane-attachment of postrationally appended proteins (Lu et al., 2015). Doing so required the design, synthesis and study of clickable *myo*-inositol substrates, during which a variety of probes containing azidoethyl tags attached at the 3-, 4-, and 5-positions were pursued to avoid the 1-, 2-, and 6-positions at which *myo*-inositol is derivatized in GPI anchors. It should be noted that the synthesis of these particular probes is quite challenging since this requires enantioselective synthesis via the desymmetrization of *myo*-inositol. While initial studies indicated that these probes were not successful, probes with esterified hydroxyl groups were explored in an effort to increase hydrophobicity in order to cross membranes, after which ester moieties can be removed by intracellular esterase enzymes. Partially acylated probes, exemplified by **4AEI** were found to be particularly effective for GPI labeling, as judged by fluorescence imaging and flow cytometry after CuAAC labeling as well as competitive inhibition through treatment with per-acetylated *myo*-inositol and treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) to detach labeled proteins.

In subsequent work, the Guo group sought a modified strategy by which they could enhance selectivity for labeling of GPI anchored proteins in cancer cells (Jaiswal et al., 2020). To do so, alternate probes were developed, exemplified by **Ac₂Lys4AIE**, in which an *N^α,N^ε*-diacetyl-L-Lysine (Ac₂Lys) moiety was linked via a self-immolating *p*-aminobenzyl alcohol (PABA) tether as a caging group at the 1-position of *myo*-inositol. This was designed such that the caging group would be removed by two enzymes, histone deacetylase (HDAC) and cathepsin L (CTSL), that are overexpressed in cancer, thereby selectively unmasking the substrate analog in diseased cells. This result was supported by flow cytometry and fluorescence imaging showing that probes of type **Ac₂Lys4AIE** exhibited enhanced labeling of MCF-7 breast cancer cells compared to normal human fibroblast cells (IMR-90), whereas probes of type **4AEI** resulted in similar labeling in both cell lines.

Our group has been interested in labeling *myo*-inositol-containing phospholipids including phosphatidylinositol (PI) and its downstream phosphatidylinositol polyphosphate (PIP_n) products, which are critical regulators of several key biological processes (Ricks et al., 2019). In this case, we avoided modification of *myo*-inositol at the 3-, 4-, and 5-positions where these compounds are phosphorylated in nature to produce the seven core PIP_n isomers, along with the 1-position as the site of phospholipid attachment. Of the remaining 2- and 6-positions, the former was selected since inositol-1,4,5-trisphosphate (IP₃) probes modified at the 2-position have been found to effectively bind IP₃ receptors (Hirata et al., 1989; Hirata et al., 1990; Ozaki et al., 1992; Watanabe et al., 1991). Therefore, probe **2API** was synthesized and studied, and found to diminish conversion of radiolabeled *myo*-inositol to PI in a cell extract PI synthase assay. It also enabled fluorescence imaging of labeled PI products in cells supported by MS studies in which tagged PI products were detected and fluorescence-based thin-layer chromatography (TLC) experiments in which dose-dependent of new spots that correlated with commercial PI and PIP_n standards were observed. In our case, probe **2API** was found to be superior to peracetylated analogs. We also observed interesting cell growth results in which increasing concentrations of probe **2API** enhanced cell growth, in contrast to decreased growth observed when raising *myo*-inositol concentrations. This could be attributed to the ability of **2API** to produce important PI lipids while perhaps not repressing phospholipid biosynthesis, which natural *myo*-inositol is known to do. In addition to these articles, other clickable inositol probes have been reported as putative substrates for metabolic labeling of inositol-containing lipids (Ausmus et al., 2020; Pasari et al., 2015).

While beyond the scope of glycerophospholipid labeling, we will note that success has also been achieved with sphingolipid probe development. For example, Cairo and co-workers developed clickable sphingomyelin probes and showed that sphingomyelinase enzymes still accept these compounds and their products of click chemistry derivatization as substrates for hydrolysis (Sandbhor et al., 2009). Penno, Thiele and co-workers developed alkynyl-1-deoxysphingolipid analogs to detect restrictions in metabolism and links to mitochondrial dysfunction (Alecú et al., 2017). Wittman and co-workers have reported clickable analogs of glycosphingolipids containing mono- and disaccharides at the headgroup (Dauner et al., 2016). Finally, incorporation of azido-sugars into liposomes, either through lipidation or

encapsulation, has also been shown to enhance metabolic labeling of carbohydrates (Shen et al., 2019).

These initial reports of lipid metabolic labeling showcase wide-ranging strategies that can be invoked to successfully label important and widely variable lipid products in order to detect and study metabolism in cells. The common theme is that careful manipulation of substrate architecture, benefitting from diminutive click tags, can enable infiltration of biosynthetic machinery to produce labeled products in cells. In certain cases enzymes can be surprisingly tolerant to substrate modifications. While these initial reports of labeling strategies have provided insights into lipid biosynthesis and trafficking, as was discussed in the prior summaries, they have also inspired numerous subsequent studies in which these techniques have been applied for a wide range of studies aimed at understanding lipid production and activity. The next section will focus on these recent applications of the lipid metabolic labeling technique.

3. Applications Involving Detection and Imaging of Labeled Lipids

The utility of lipid metabolic labeling techniques for tracking metabolism and answering biological questions was already demonstrated in the first reports in this field. Since those initial studies, demonstrations of the applicability of this approach have rapidly progressed, and the examples that will be subsequently discussed showcase the power of metabolic labeling for studying biological processes at the molecular, cellular, and organismal levels. A primary benefit of the lipid metabolic movement is its versatility, as this technique enables a plethora of studies in which wide-ranging tags can be introduced onto labeled lipid products for detection and manipulation by a variety of techniques. Labeling of PC lipids using choline probes **PCho** and **AECho**, the first of this type to be reported, has been particularly mined for applications.

3.1. Investigations of Lipid Metabolism, Trafficking, Localization, and Protein Binding

An intrinsic benefit of lipid metabolic labeling is that it enables direct imaging of the synthesis and localization of lipid products, which is invaluable for probing the complex processes of lipid metabolism and trafficking. Multiple reports have showcased the efficacy of labeling strategies for elucidating these complex processes. Cairo and co-workers followed up on lipid metabolic labeling using **PCho**, **AECho** and a ketone- containing choline (Li et al., 2014). In doing so, they explored various live cell imaging protocols and showed that lipid dynamics could be studied via fluorescence recovery by photobleaching, and that cells could be attached onto functionalized surfaces by click chemistry. Lang, Thiele, and co-workers used PC imaging by **PCho** to rule out exchange of lipid content at contact sites between the ER and PM (Merklinger et al., 2016). In work that showed that lipid droplets (LDs) in nuclei of hepatocytes are derived from apiloprotein B-free luminal LDs, Fujimoto and co-workers used fluorescence imaging of **PCho** labeling to demonstrate that perilipin-3-V5 decreased PC synthesis, which was reversed by a modified protein (perilipin-3-NES-V5) containing an appended nuclear exclusion signal driving localization to the cytoplasm (Soltysik et al., 2019). **PCho** labeling has also been used to image the localization of PC biosynthesis during mitosis (Sawicki et al., 2019). This helped show that

PC and FA synthesized at the mitotic exit end up in the nuclear envelope, and that FA and PC biosynthesis, but not decreases in LPC, are necessary for the cell cycle to proceed past the G₂ and M phases. These results pointed to FA biosynthesis as a means for anti-cancer intervention.

Schrack and co-workers investigated fluorescence imaging of PC labeling in plants by subjecting *Arabidopsis thaliana* seedlings to propargylcholine probe **PCho** followed by CuAAC (Paper et al., 2018). They initially observed no difference between plants grown in the presence or absence of **PCho**. Seedlings and mature plants subjected to labeling and confocal microscopy exhibited fluorescence in all tissues examined, with especially strong signal observed in the roots and guard cells of the epidermis of leaves, respectively. This signal was abrogated by treatment with PLC, as expected due to hydrolysis and removal of the click-tagged choline headgroup. In fluorescence colocalization studies, fluorescence-tagged PC was found to be primarily excluded from the cell wall and nuclei, but present in regions including the ER, PM, and tonoplast, along with variable inclusion in mitochondria. MS studies comparing labeled to unlabeled PC also supported greater mole percent incorporation in the roots (~50%) compared to seedlings, leaves, stems, cotyledons, and siliques (~18-28%)

Recently, Griese and co-workers employed PC labeling using **PCho** to study the activity of ATP-binding cassette sub-family A member 3 (ABCA3) in the formation and trafficking of lipids into the lamellar body (LB), processes that are dysregulated in chronic interstitial lung diseases (Li et al., 2019b). This labeling protocol was first validated for the fluorescence imaging of PC lipid products in ABCA3+ vesicles, with all chosen vesicles labeled after 24 hours. Next, transport of tagged PC products was found to be ATP-dependent based on the decrease in fluorescence signal upon treatment with the inhibitor orthovanadate.

Experiments performed in the presence of choline kinase inhibitors MN58B and miltefosine (MLF) indicated that PC synthesis took place in the first 12 hours of experiments in which ABCA3+ vesicle labeling was reduced, but this was restored when inhibitors were introduced after that time. Additionally, two mutants of ABCA3 known to correlate with neonatal surfactant deficiency were found to result in smaller ABCA3+ vesicles than wild type, although the time curves for lipid synthesis and transportation were found to be similar. This report highlights the utility of lipid metabolic labeling for elucidating the details of disease-related defects in protein machinery that controls lipid synthesis and transport.

In an intriguing example, Yao, Wenk and co-workers applied lipid metabolic labeling to aid in the global identification of lipid-binding proteins (Wang et al., 2017). A recent strategy to this end has entailed the development of bifunctional lipid probes containing both a cross-linking group by which the probe becomes attached to bound proteins as well as a secondary tag that enables their enrichment and identification (Best, 2014; Best et al., 2011; Laguerre and Schultz, 2018). A common concern with this approach is that the probe itself could enter metabolism to generate modified probes that correspond to different lipids. These modified probes could then label proteins that are not bound by the original probe target, leading to false positives. This article reported an ingenious solution to this issue by exploiting metabolic labeling using **PCho** to introduce the clickable alkyne tag for post-derivatization

(Scheme 3). Therefore, while downstream probe analogs could still be produced and label proteins, the clickable tag used for postderivatization would only be present for PC probe products resulting from metabolic labeling. Using this approach, FAs containing diazirine crosslinking groups were treated alongside **PCho** to label and identify a number of PC-binding proteins.

Liu and co-workers exploited PC lipid metabolic labeling as a tool for imaging the production and cell entry of exosomes, which are important mediators of communication and transport between cells. This was performed through both **PCho** derivatization of **PC** (Zhang et al., 2018b) as well as PLD-mediated **Hexynol** (Jiang et al., 2020) labeling of PA. To do so, exosomes of larger sizes derived from breast cancer cell lines were enriched by centrifugation followed by fluorescence labeling. In the former study, exosomes produced by different cell lines were found to exhibit distinct variations in uptake in different organs. In the latter study, comparison of PA metabolic labeling with Dil staining showed that fluorophores that were not covalently appended to lipids were successfully removed by washes, prior to which the two fluorescence signals were colocalized in exosomes. Overall, transmission electron microscopy (TEM), dynamic light scattering (DLS), Western blot, and cell migration studies showed that labeling did not affect exosome morphology, size, zeta potential, protein inclusion in exosomes, cell scratch healing efficacy, and promotion of cell proliferation. Studies also showed this approach to be superior to Dil dye incorporation in terms of the stability of the fluorescence signal over time. Finally, systematic fluorescence microscopy studies enabled a series of insights regarding EV entry into RAW264.7 cells, including that this occurs in a time-dependent manner (beginning at ~1 hour and ending after ~8 hours) and is driven by interactions with filopodia and subsequently the PM. Temperature-dependence was also a sign of active endocytosis events, and a panel of selective inhibitors indicated that EV cell entry was primarily driven by micropinocytosis and phagocytosis.

3.2. Lipid Metabolic Labeling for Viral Studies

A prominent family of targets has involved viruses, either by labeling of viral particles themselves or by elucidating changes in lipid metabolism in host cells caused by viral infection. Xie and co-workers have reported several strategies showcasing the ability to label lipids present within materials ranging from viruses to nanoparticles for drug delivery, efforts that have been recently reviewed (Huang et al., 2020). For the former, attention was turned to metabolic production of azide-tagged PC in host cells using **AECho** for labeling by SPAAC. PC was selected since it is the most abundant lipid in mammalian membranes and is enriched in the outer PM, providing suitable presentation of the tag. This approach was used to label vaccinia virus (VACV) membranes by exploiting the phenomenon of host-derived viral membranes (Huang et al., 2013). The resulting viral particles showed similar titers to unlabeled control virus and induced the cytopathic effect. This approach was combined with viral nucleic acid imaging to achieve two-color labeling of the virus, which enabled single particle tracking. Systematic studies showed that VACV Tiantan strain entered vero cells through pinocytosis rather than membrane fusion, which was dependent on ATP and actin but not on caveolin. Wang and co-workers showed that the measles virus could be decorated with quantum dots by SPAAC following **PCho** treatment (Zhao et al.,

2016). Finally, Cai and co-workers showed this approach to be effective for the labeling and imaging of H5N1 pseudotype virus in a manner that preserves virus infectivity and immunogenicity (Pan et al., 2017).

Mota and co-workers studied **PCho** labeling in malaria and found that *Plasmodia* hijacked host enzymes to enhance the biosynthesis of PC as well as neutral lipids. The upregulated PC products were found to be taken up by the parasites and localized to parasite PMs, parasitophorous vacuole membrane, and exo-erythrocytic forms, which were determined to be vital for parasite survival (Itoe et al., 2014). Wang and co-workers used **PCho** labeling to show that all positive-strand RNA viruses, including poliovirus, hepatitis C virus, and brome mosaic virus, exploit host enzymes that synthesize PC to support viral replication (Zhang et al., 2016). In addition, it has been demonstrated that the enzyme CTP:phosphocholine cytidyltransferase (CTP:PCCT) translocates from the nucleus to the cytoplasm upon infection of poliovirus, which results in conversion of neutral lipids from lipid droplets (LDs) into phospholipids (Viktorova et al., 2018). In doing so, Belov and co-workers used **PCho** to demonstrate that inhibition of LD-associated lipases by diethylumbelliferyl phosphate (DEUP) severely inhibited phospholipid synthesis in infected cells, while inhibitors of other phospholipid biosynthesis pathways did not.

3.3. Applications of Lipid Metabolic Labeling to Cancer Imaging and Study

Due to the important roles that lipids play in cell growth and proliferation, applications of lipid metabolic labeling to image and study cancer onset have emerged, as have potential avenues for therapeutic intervention. Nitin and co-workers have applied **PCho** labeling for a series of studies aimed at cancer imaging and treatment. For example, this technique has been used to develop an optical molecular imaging approach to visualize changes associated with oral neoplasia. Here it was found that cell mean fluorescence intensities resulting from **PCho** fluorescence labeling of neoplastic tissues were 4-5-fold greater than normal samples (Luo et al., 2013). This labeling protocol was also used to determine the effects of different anti-cancer drugs on choline metabolism, and indicated that the inhibitors cisplatin (Luo et al., 2012), gefitinib, and carboplatin (Luo et al., 2016) were detectable based on decreased **PCho** uptake. The latter cases showcased decreased labeling in drug-sensitive cell lines while not in drug-resistant cell lines. The probe **AECho** has also been exploited to radiolabel PC lipids through SPAAC using ^{99m}Tc chelates (Chen and Chu, 2016). As will be discussed in the next section, the Xie group has been interested in developing clickable nanoparticles for drug delivery using lipid metabolic labeling strategies. Recently, they exploited the enhanced presentation of labeled PC products resulting from **AECho** treatment on cancer cell surfaces for the targeting of DBCO-containing nanoparticles (Lu et al., 2020). This report showcases exciting prospects for the use of metabolic labeling techniques for targeted drug delivery.

3.4. Studies Involving Nanoparticle Drug Delivery Platforms

The Xie group has also been interested in using this technique to engineer nanoparticle surfaces using natural or metabolically labeled lipids for cell targeting in biomedical delivery applications. This approach was initially used to modify magnetic nanoclusters (MNCs) that were camouflaged with cancer cell membranes to develop

immunomagnetsomes (IMs) (Xiong et al., 2016). This strategy was applied to detect circulating tumor cells (CTC) by decorating IMs with membranes containing metabolically labeled azido-PC followed by SPAAC to introduce the antibody of epithelial cell adhesion molecule (EpCAM) as a CTC biomarker. This enabled capture of ~90% of CTCs from blood in 15 minutes. This was later enhanced by switching to platelet-leukocyte membranes (Rao et al., 2018) and through detection using a microfluidic system (Zhang et al., 2019b).

Similar nanoparticles camouflaged with clickable PC have been applied for different goals, highlighting the versatility of this strategy. Adsorption of siRNA onto MNCs followed by decoration of azide-tagged PC with RGD peptides enabled a highly effective gene delivery platform that delivers siRNA directly into the cytoplasm (Zhang et al., 2018a). Decoration of MNCs with T-cell stimulating peptides produced artificial antigen-presenting cells (aAPCs) that were highly effective for stimulating the production of CD8+ T cells (Nie et al., 2019; Zhang et al., 2017). MNCs were also developed to trigger ferroptosis in cancer cells by decorating the membrane with PD-1 antibody while incorporating the hydrophobic TGF- β inhibitor Pa, generating a macrophage-mediated immune response (Zhang et al., 2019a). Finally, an approach to a cancer vaccine was developed by appending anti-CD205 antibodies to MNPs onto which the Toll-like receptor CpG-ODN was adsorbed. The former enhanced recognition of CD8+ cells, while the latter was used to activate an immune response (Zhu et al., 2017).

3.5. Lipid Metabolic Labeling in Conjunction with Raman Spectroscopy Techniques

Another tool for lipid detection that has been enhanced through lipid metabolic labeling is the technique of stimulated Raman scattering (SRS), which enables high-sensitivity detection of biomolecules such as lipids without requiring bulky dyes to be appended (Hu et al., 2019; Liu et al., 2020; Wei et al., 2016). Expanding beyond the use of traditional isotope labels (Matthaus et al., 2012; Shi et al., 2018), the alkyne moiety in particular is beneficial since it produces Raman peaks that correlate with silent regions of signals produced by cells, further enhancing resolution. Min and co-workers pursued lipid imaging in reports that also included analysis of labeled nucleic acids and proteins (Hu et al., 2016; Wei et al., 2014). Here, propargylcholine probe **PCho** enabled the labeling of PC lipids in cellular membranes of hippocampal neurons while treatment of *C. elegans* with tagged fatty acid 17-octadecynoic acid was found to primarily result in incorporation into LDs in the form of triglycerides. Additionally, Raman studies of **PCho** (Zhang and Min, 2017) and alkyne-tagged fatty acid probes (Hong et al., 2014; Li et al., 2019a) have been used to investigate lipid metabolism and cholesterol probes have been used to assess cholesterol storage (Lee et al., 2015). Lipid metabolic labeling has also been detected using the technique of surface-enhanced Raman scattering (SERS) (Xiao et al., 2014).

3.6. Electron Microscopy Efforts Exploiting Labeled Lipids

Lipid metabolic labeling in conjunction with click chemistry derivatization has also recently emerged as a powerful tool for conducting electron microscopy imaging experiments. Fujimoto, Salic, and co-workers developed a technique of this type to analyze the distribution of PC lipids in the inner and outer leaflets of different membranes using a freeze-fracture protocol employing **PCho** (Iyoshi et al., 2014). Following metabolic labeling

of PC, CuAAC was used to introduce biotin, which was then detected using antibiotin primary antibodies followed by colloidal gold-tagged secondary antibodies. This approach facilitated imaging of membranes at a resolution in which membrane leaflets could be differentiated. This enabled the authors to observe surprising results; budding yeast Golgi and PM showed asymmetry with PC exhibiting cytoplasmic leaflet dominance, while other organelles were found to be evenly distributed. In mammalian cells, the PM instead showed symmetric distributions. Lipid subcellular localization is critical for controlling lipid activity and membrane properties, and these results provide important insights into these phenomena at the molecular level as well as insights into variations between organisms.

EM has also been enhanced for lipids and other molecules by using click chemistry-based post-derivatization to introduce tags that catalyze the photooxidation-based polymerization of diaminobenzene (DAB) for detection purposes (Ngo et al., 2016). This has been performed through PC labeling using **AEcho** followed by SPAAC to introduce IRDye700X that acts as an antenna to catalyze this process. This enabled imaging of intracellular membranes, and particularly the ER membrane due to its high PC content. This technique also enabled visualization of detailed features such as ER-mitochondrial contacts and mitochondrial folds.

4.0. Conclusions

This review article showcases the vast possibilities for biological studies that are enabled by lipid metabolic labeling. Despite this, it is clear that lipid studies of this type have lagged behind the metabolic labeling of other molecules. Of the pathways shown in Scheme 1, successful labeling strategies have only been reported for a handful of these molecules. One might surmise that this coincides with the woeful history of the lipid molecule as being the biomolecule that is most commonly overlooked. However, upon closer inspection, intrinsic challenges of lipid metabolic labeling strategies emerge. First off, the fact that lipid molecules are interconverted among one another through a web of transformations leads to problems when one attempts to label one particular lipid. For example, in our investigation of PI labeling using **2API**, we obtained evidence suggesting the additional labeling of downstream PIP_n molecules. This makes sense since the *myo*-inositol headgroup is retained among these molecules, but as a result it is not clear how one would selectively target one particular inositol lipid family member and not the others. Another concern is the simplicity of the substrates that are used to introduce the headgroups, such as glycerol, ethanolamine, and serine, which leads to concern about how these diminutive molecules can be modified to produce viable analogs for metabolic labeling. Nevertheless, the recent successes in labeling multiple lipids promotes enthusiasm that that these strategies can work in the context of lipids. In particular, the successful metabolic labeling of PA, which is formed as a result of the nucleophilic attack of water, provides confidence that lipid labeling can be tackled with some ingenuity.

Concerns about the challenges associated with developing successful lipid labeling strategies are mitigated by the vast array of applications that we now see are enabled by this technology. Since the labeling of PC lipid products using clickable tags was the first approach to be reported by Salic and co-workers, this technique has inspired the greatest

amount of subsequent work thus far. In the slightly more than 10 years since that protocol was initially reported, it has been applied by a number of labs for myriad studies including: elucidation of lipid metabolism, trafficking, subcellular localization, and identification of protein-lipid binding interactions, exosome tracking, detection and investigation of the mechanism of viral particles, cancer imaging, study of disease onset, characterization of inhibitor activity, and tracking and targeting of nanoparticle-based drug delivery platforms. These varied studies showcase the immense versatility of this approach. In addition, lipids labeled by these strategies have been detected and investigated using a range of techniques including fluorescence spectroscopy and microscopy, mass spectrometry, chromatography, different Raman spectroscopy techniques, and electron microscopy. All of this work points to a bright future for lipid metabolic labeling that will be further enhanced by the development of strategies for labeling other glycerophospholipid molecules based on their head groups.

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Clickable substrate analog

Labeled lipid products

Labeled PC products

Labeled PA products

Figure 1. Metabolic labeling strategies for labeling PC and PA using clickable substrate analogs.

Figure 2.
Strategies for metabolic labeling of *myo*-inositol-containing phospholipids.

Scheme 1.

Workflow for lipid metabolic labeling. Derivatized substrate analogs containing clickable groups such as azide, alkyne (not shown) or *trans*-cyclooctene can be fed to cells to enter biosynthetic pathways and produce tagged products. These products can be derivatized via click reactions including CuAAC, SPAAC, the Staudinger Ligation, and IEDDA to introduce reporter groups such as fluorescent dyes, thereby enabling detection of products by a range of techniques exemplified by fluorescence microscopy, MS and TLC/HPLC.

Scheme 2.

Simplified scheme for metabolic interconversions of glycerophospholipids in mammals in order to understand pathways that have been exploited for lipid metabolic labeling. Lipid tails are generalized to denote mixtures of acyl chain lengths and unsaturation.

Scheme 3.

Approach using metabolic labeling to produce specific bifunctional PC probes for the global identification of PC-binding proteins. Note: the diazirine-containing acyl chain could be incorporated at different positions in labeled products.