**Analysis of the regulation of surfactant phosphatidylcholine metabolism using stable isotopes.**

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**Abstract**

The pathways and mechanisms that regulate pulmonary surfactant synthesis, processing, secretion and catabolism have been extensively characterised using classical biochemical and analytical approaches. These have constructed a model, largely in experimental animals, for surfactant phospholipid metabolism in the alveolar epithelial cell whereby phospholipid synthesised on the endoplasmic reticulum is selectively transported to lamellar body storage vesicles, where it is subsequently processed before secretion into the alveolus. Surfactant phospholipid is a complex mixture of individual molecular species defined by the combination of esterified fatty acid groups and a comprehensive description of surfactant phospholipid metabolism requires consideration of the interactions between such molecular species. However, until recently lipid analytical techniques have not kept pace with the considerable advances in understanding of the enzymology and molecular biology of surfactant metabolism. Refinements in electrospray ionisation mass spectrometry (ESI-MS) can now provide very sensitive platforms for the rapid characterisation of surfactant phospholipid composition in molecular detail. The combination of ESI-MS and administration of phospholipid substrates labelled with stable isotopes extends this analytical approach to the quantification of synthesis and turnover of individual molecular species of surfactant phospholipid. As this methodology does not involve radioactivity, it is ideally suited to application in clinical studies. This review will provide an overview of the metabolic processes that regulate the molecular specificity of surfactant phosphatidylcholine together with examples of how the application of stable isotope technologies *in vivo* has, for the first time, begun to explore regulation of the molecular specificity of surfactant synthesis in human subjects.

**Key words**

Lung surfactant metabolism; mass spectrometry; stable isotopes; phosphatidylcholine

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1. **Introduction**

The basic pathways for surfactant synthesis and secretion were established over thirty years ago, based on extensive studies using radioisotope-labelled substrates to monitor phospholipid metabolism in experimental animals both *in vivo* and *in vitro*. While these studies provided very valuable information that still underpin current concepts of surfactant lipid metabolism, they have inherent limitations. In common with cell membranes, surfactant phospholipids are complex mixtures of individual molecular species defined by the combination of fatty acid groups esterified to the glycerol backbone of the molecule. Such molecular species are the biologically-relevant entities that determine surfactant function and, consequently, their analysis is critically important to develop comprehensive models of surfactant metabolism. The major phosphatidylcholine (PC) species of human lung surfactant is dipalmitoyl PC (DPPC or PC16:0/16:0), which is widely accepted as the principal surface tension-lowering component. It is worth noting, however, that mixtures based on monounsaturated PC species can generate very low surface tension values under appropriate conditions of surface film compression (Crane and Hall, 2001) and that DPPC is not universally the major component of surfactant. For instance, DPPC is largely replaced by the monounsaturated PC species palmitoylpalmitoleoyl PC (PC16:0/16:1) in a number of fast breathing and in some heterothermic mammals (Lang et al., 2005) and by the shorter chain saturated species palmitoylmyristoyl PC (PC16:0/14:0) in sucking rats, due the high content of myristic acid in rat milk (Pynn et al., 2010). Moreover, this diversity of surfactant lipid composition is equally applicable to human development. The immature epithelial cells in the lungs of the first trimester fetus can be differentiated in culture under appropriate hormonal treatment into cells that morphologically resemble type II alveolar epithelial cells (ATII cells) (Gonzales et al., 2002). These cells contain lamellar bodies characteristic of surfactant storage before secretion, express surfactant-specific proteins and secrete functional surfactant. This differentiation processes is paralleled by cell-specific changes to phospholipid composition, but with enrichment of PC16:0/16:1 and not of DPPC (Postle et al., 2006). However, while the ATII cell can package a variety of PC species into lamellar bodies destined for surfactant secretion, the sum of PC16:0/16:0, PC16:0/14:0 and PC16:0/16:1 almost invariably contribute approximately 70% of surfactant PC, across all animal species and experimental conditions. In metabolic terms, these short chain species can be regarded as surfactant-specific, while unsaturated PC species with longer acyl chain fatty acids such as oleate, linoleate and arachidonate are more associated with the cellular membranes. Consequently, surfactant phospholipid metabolism should be analysed by methodologies that can distinguish between these diverse molecular species.

Historically, detailed analysis of phospholipid molecular species compositions has been a laborious process, typically requiring derivatisation of their diacylglycerol moieties after hydrolysis with phospholipase C followed by HPLC with either UV or fluorescence detection (Robins and Patton, 1986; Takamura and Kito, 1991). Analysis of the molecular specificity of PC synthesis by monitoring incorporations of radioactive precursors is equally laborious, involving collection of HPLC fractions followed by scintillation counting (Caesar et al., 1991) or on-line radioactivity monitoring . Consequently, many experimental animal and cell culture studies of surfactant phospholipid metabolism have relied on oxidation of unsaturated PC molecules by osmium tetroxide (Mason et al., 1976) to provide an analysis of total disaturated PC (satPC or DSPC) as a surrogate for DPPC. However, DSPC is a mixture of PC species in addition to DPPC, largely PC16:0/14:0, but does not include PC16:0/18:1 and cannot provide any insights into the metabolism of non-surfactant compared with surfactant-specific PC species. While much of our understanding of surfactant metabolism is based on this analytical approach, it has a number of limitations. First, DSPC is not specific for DPPC. Second, less than 50% of surfactant DPPC is synthesised *de novo* by the CDP:choline pathway (Caesar et al., 1991; Mason and Nellenbogen, 1984; Post et al., 1983; Stymne and Stobart, 1985), with the remainder being generated through acyl remodelling by the Land’s cycle (see below). Analysis of acyl remodelling requires detailed determination of interactions between individual PC molecular species, but this cannot be provided by satPC analysis.

Modern developments in mass spectrometry (MS), especially electrospray ionisation MS (ESI-MS), have provided new sensitive tools to probe the complexity of surfactant metabolism in greater detail. Additionally, as the use of radioactivity is not compatible with clinical studies, very little information was available about the regulation of human surfactant phospholipid metabolism. Introduction of substrate probes labelled with non-radioactive stable isotopes, combined with a variety of MS instrumentation and techniques, now provides a ready experimental approach to probe surfactant metabolism in the clinical setting. This review will first outline aspects of the biochemistry regulating surfactant composition and synthesis and then highlight recent advances in understanding of surfactant synthesis and turnover, based on ESI-MS techniques.

**Fatty acid synthesis and supply**

The fatty acids that become incorporated into surfactant phospholipids are predominantly either saturated or monounsaturated and, as such, can either be synthesised *de novo* or supplied from the diet. Palmitate is the end product of fatty acid synthase, and can be readily converted into monounsaturated fatty acids such as palmitoleate or oleate by the processes of desaturation and chain elongation. More unsaturated fatty acids containing two or more unsaturated double bonds must be provided from dietary sources. The balance between fatty acid synthesis *in situ* and fatty acid delivery from the circulation is not well defined; isolated alveolar type II (ATII) cells can readily synthesise surfactant fatty acids but considerable evidence in rodent lung demonstrates palmitate synthesis by the lipofibroblast and subsequent paracrine delivery to the ATII cell (Nunez and Torday, 1995; Torday and Rehan, 2011). While the lipofibroblast has been identified in human lung (Rehan et al., 2006), its role in the supply of lipid substrate for surfactant synthesis has not yet been established.

The importance of lipid supply from the lipofibroblast to surfactant lipid synthesis was clearly demonstrated in transgenic mice by modulation of the sterol regulatory element-binding proteins (SREBP) that regulate, in part, lipogenesis in the lungs. Four isoforms of SREBP (SREBP-1a, SREBP-1c, SREBP-2 and SREBP-2g are synthesised as inactive precursors that bind to SREBP cleavage-activating protein (SCAP) in the endoplasmic reticulum (Dong et al., 2012). The interaction of SCAP and SREBP is best documented for regulation of cholesterol synthesis. SCAP acts a lipid sensor, and in conditions of membrane cholesterol depletion, transports the SREBPS from the endoplasmic reticulum to the Golgi where they are subject to two proteolytic cleavage and release of the biologically-active transcription factor, nuclear SREBP (nSREBP). nSREBPs then translocate to the nucleus where they bind to sterol response elements (SRE) in promoter regions of target genes, resulting, among other consequences, in increased cholesterol synthesis. SREBPS, especially SREBP-1c, are also core to the regulation of fatty acid and phospholipid synthesis in the lung and provide a good example of the cellular interdependence of surfactant phospholipid synthesis in the lungs. Conditional deletion of SCAP in respiratory epithelial cells decreased lung SREBP expression, resulting in decreased phospholipid synthesis in isolated ATII cells (Besnard et al., 2009). Importantly, there was no apparent phenotype or altered BALF lipid composition in *ScapΔ/Δ* mice *in vivo*, due to compensatory enhancement of PC synthesis by the lipofibroblast and paracrine supply of PC and fatty acids from lipofibroblast to ATII cell.

The complexity and tight regulation of lung surfactant lipid synthesis was further demonstrated by conditional deletion in ATII cells of insulin-induced gene protein-2 (Insig-2) in mice with germ line deletion of Insig-1 (Plantier et al., 2012). Insigs anchor the SCAP-SREBP complex to the endoplasmic reticulum in a lipid dependent fashion and inhibit SREBP activation and lipid synthesis. *Isig1/2Δ/Δ* mice exhibited lung accumulation of the neutral lipids triacylglycerol and cholesterol esters. This accumulation was specific for the subset of *Isig1/2Δ/Δ* ATII cells, as the subset of ATII cells in the same lungs that expressed *Insig1* had a normal lipid phenotype. Importantly, however, this lipotoxicity of neutral lipid had no effect on lung PC synthesis, surfactant lipid composition or surfactant function, demonstrating the significant redundancy in the regulation of surfactant lipid composition. Importantly, this observation illustrates the tight regulation of PC metabolism even under conditions of increased substrate fatty acid supply in ATII cells**.**

1. **The regulated enzymology of PC synthesis by ATII cells**

PC is synthesised by the CDP:choline pathway in ATII cells, involving the sequential activities of choline kinase, CTP:cholinephosphate cytidylyltransferase (CCT) and cholinephosphotransferase (CPT). Considerable evidence indicates that CCT is the major regulatory enzyme of de novo PC synthesis in lungs and ATII cells (Post et al., 1984b; Spragg and Li, 2000; Tesan et al., 1985), although CPT exerts a regulatory role, for instance when lysoPC acyltansferase 1 (LPCAT1) is over-expressed in epithelial cells. This leads to increased PC synthesis from lysoPC, with PC synthesis *de novo* decreased by enhancing CPT ubiquitination and degradation (Butler and Mallampalli, 2010). Concentration analyses of CDP:choline pathway intermediates supports a regulatory role for CCT, as its substrate choline phosphate present at higher concentration than its product CDP:choline (Post et al., 1984a). The CCTα isoform is predominantly expressed ATII cells (Wang et al., 2005) and is regulated both by protein expression and by activation. Germline deletion of CCTα is embryologically lethal (Wang et al., 2005), while inducible deletion of the enzyme at embryological day 7.5 permitted normal lung morphogenesis and development but was neonatally lethal due to lack of surfactant PC synthesis (Tian et al., 2007). CCT expression and PC synthesis is enhanced by antenatal and postnatal administration of PPARγ agonists (Rehan et al., 2010), cell stretch (Ren et al., 2009), vasoactive intestinal peptide (Li et al., 2007), sphingosine (Ryan et al., 2004) and keratinocyte growth factor (Gesche et al., 2011; Yano et al., 2000). In contrast, lung CCT expression is decreased by TGFβ-1 (Qiu et al., 2011), infection with Pseudomonas aeruginosa (Wu et al., 2007) and dietary Zn deficiency (Gomez et al., 2006),

1. **Phospholipid trafficking in ATII cells**

A number of possible routes have been proposed for transport to lamellar bodies of phospholipid synthesised on the endoplasmic reticulum of ATII cells. Early autoradiography studies using 3[H] labelled choline suggested that newly synthesised surfactant PC is trafficked as vesicles from endoplasmic reticulum to the lamellar bodies via the Golgi (Chevalier and Collet, 1972), but this suggestion has been questioned as treatment with Brefeldin A blocked protein trafficking through the Golgi to lamellar bodies but had no effect on phospholipid transport (Osanai et al., 2001). A possible explanation is provided by the concept that the Golgi may be bypassed by a mechanism involving direct transfer of surfactant lipids from endoplasmic reticulum to LBs at specific membrane contact sites (Perez-Gil and Weaver, 2010). Alternatively, a number of phospholipid transfer proteins have been purified and found to be enriched in ATII cells (Lumb, 1989; Pool et al., 1983; Post et al., 1980), the best characterised of which is PC-TP/StarD2, a member of the START (steroidogenic acute regulatory protein (STAR)-related lipid transfer) domain family of proteins. Mouse lung expression of PC-TP/StarD2 is maximal in the late fetal, early neonatal periods in parallel with surfactant maturation but lamellar body and surfactant phospholipid compositions were unaltered in *Pc-tp*-/- mice (Kanno et al., 2007; Van Helvoort et al., 1999) suggesting either that StarD2 does not facilitate lung surfactant PC trafficking or there are additional compensatory mechanisms. Considerable evidence indicates that alternative phospholipid transport mechanisms in addition to lamellar body supply, with a proportion of newly-synthesised phospholipid clearly trafficked to the basolateral membrane for subsequent export to the circulation or lymphatic system, mediated by ABCA1 transfer to ApoA1. ABCA1 is located in the basolateral membrane of ATII cells and can be pharmacologically induced by incubation with either 9-cis-retinoic acid or 22-OH-cholesterol (Bortnick et al., 2003). This activation was characterised by increased basolateral efflux of PC to ApoA1, with associated decreased secretagogue-stimulated phospholipid secretion from the apical membrane. The interdependence of the basolateral and apical secretion routes in ATII cells was further illustrated by the alveolar lipoproteinosis that develops in ABCA1-/- mice, characterised by increased alveolar concentrations of both PC and especially of cholesterol esters (Bates et al., 2005). The physiological, and potentially clinical, significance of these observations was clearly illustrated by the activation of ABCA1 and enhanced basolateral phospholipid transport in AT11 cells infected with adenovirus (Miakotina et al., 2007) or Pseudomonas aeuriginosa (Agassandian et al., 2007).

1. **Metabolic regulation of lung and alveolar PC concentration**

Under equilibrium conditions, with a constant concentration of PC in the lungs, the rates of PC synthesis and catabolism must balance, otherwise PC would either accumulate or become depleted. The various pathways that combine to regulate surfactant lipid metabolism in the ATII cell are summarised in Figure 1. Regulation of ABCA1 is one mechanism for achieving such a balance, for instance in ATII cells over-expressing CCTα (Zhou et al., 2004). Such cells have increased CCT activity and stimulated flux of PC synthesis, but have no accumulation of PC content due to a compensatory increased ABCA1-mediated basolateral PC export to ApoA1. Conversely, the importance of surfactant lipid catabolism and removal by alveolar macrophages is demonstrated by the progressive accumulation of PC and other lipids in the alveoli of patients with alveolar lipoproteinosis. Although a wide spectrum of conditions, the most common underlying pathology is development of autoantibodies against GM-CSF, resulting in failure of terminal differentiation of alveolar macrophages and impaired catabolism of surfactant phospholipid (Carey and Trapnell, 2010). A comparable pathology was seen in transgenic mouse models, with the GM-CSF-/- developing a more severe phenotype than the GM-CSF β-chain receptor-/- mouse (Reed et al., 2000). Surfactant phospholipid catabolism was impaired in both mice, but with unchanged rates of surfactant secretion compared with control mice (Ikegami et al., 1996). A central role for the peroxisomal proliferator activator receptor γ (PPARγ) in lung surfactant phospholipid catabolism is shown by the PPARγ-/- mice, which replicate the phenotype of GM-CSF-/- mice (Baker et al., 2010). One possible mechanism of this effect is the associated decreased expression of ABCG1, responsible for export of PC from the alveolar macrophages to HDL, in the PPARγ-/- mice. This mechanism was supported by evidence that lentivirus mediated transfection of either PPARγ (Malur et al., 2011a) or ABCG1 (Malur et al., 2011b) targeted to alveolar macrophages reverses lipid accumulation in GM-CSF-/- mice. Targeted over-expression of GM-CSF in the respiratory epithelium effectively restored clearance of surfactant phospholipid, in this case at an increased rate compared with control mice (Ikegami et al., 1997). Depletion of rat alveolar macrophage numbers by treatment with dichoromethylene diphosphonic acid-loaded liposomes caused a significant increase in alveolar surfactant concentration, further emphasising a central role of lipid catabolism by this cell type in the regulation of surfactant phospholipid pool size (Forbes et al., 2007). Moreover, these studies demonstrate, in at least these mouse models, that increased concentration of phospholipids in the alveolus do not directly result in feedback inhibition of either phospholipid synthesis or surfactant secretion. Intra-alveolar accumulation of surfactant phospholipid and protein is also observed in a number of other mouse models, including the surfactant protein D null mouse (SP-D-/-), but in this case there was no apparent defect in alveolar macrophage catabolism of instilled radiolabelled surfactant PC (Ikegami et al., 2001) despite decreased overall surfactant phospholipid catabolism with unchanged surfactant PC synthesis and secretion (Postle et al., 2011).

A critical role of alveolar macrophages has also been identified in the adaptations of lung surfactant metabolism to air breathing in the post-natal lung. Mammals which exhibit a significant period of lung maturation in utero, as opposed to rodents such as rats and mice where the bulk of lung development is post-natal, accumulate significant stores of intracellular stored surfactant in late gestation (Jackson et al., 1988). Newborn rabbits have a reduced rate of surfactant PC synthesis combined with a significantly decreased rate of surfactant PC catabolism compared with older animals (Jacobs et al., 1982), leading to sustained high surfactant concentrations during the perinatal period. Differential bronchoalveolar lavage cell analysis in postnatal rabbits (Zeligs et al., 1977) and monkeys (Jackson et al., 1988) show a paucity of mature alveolar macrophages at birth but the presence of small numbers of monocyte-like cells. There then follows a rapid increase in cell number and size in the first few postnatal days to resemble adult alveolar macrophages morphologically and functionally, which inversely mirrors the progressive postnatal decline in the initial high concentration of alveolar surfactant concentration. The comparable progressive decline of bronchoalveolar lavage phosphatidylcholine concentration in diagnostic bronchoscopies of children over the first three years of age suggests that alveolar macrophages may have a similar important role in the regulation surfactant concentration in human infants (Mander et al., 1997).

1. **Molecular specificity of surfactant phospholipid synthesis and composition**

The molecular specificity of surfactant phospholipid is the net result of a number of interacting metabolic processes. PC in the endoplasmic reticulum of ATII cell is initially synthesised *de novo* by the CDP:choline pathway predominantly as unsaturated species, such as palmitoyllinoleoylPC (PC16:0/18:2) and palmitoyloleoyl PC (PC16:0/18:1). This unsaturated PC is then modified by at least three separate processes. The first is acyl remodelling, which involves phospholipase A2-mediated removal of the unsaturated fatty acid at the *sn*-2 position of the glycerol backbone followed by re-esterification with palmitoyl CoA to generate DPPC (Post et al., 1983). The second involves ABCA3-mediated transport of selected phospholipid molecular species into lamellar bodies (Garmany et al., 2006; Mulugeta et al., 2002), predominately saturated PC and phosphatidylglycerol species. The third, which is often overlooked, is the ABCA1-mediated export of unsaturated PC species into the circulation across the basolateral membrane of the ATII cell (Bates et al., 2005; Zhou et al., 2004). These three mechanisms interact to regulate the enrichment of disaturated PC in lung surfactant.

The PLA2 responsible for initial hydrolysis of *de novo* synthesised PC in acyl remodelling has been identified as a lysosomal calcium independent enzyme (Fisher et al., 2005). The most widely studied enzyme is peroxiredoxin-6 (Prdx6), which is bi-functional, with one active site involved in anti-oxidant activity and the other has PLA2 activity. Prdx6 is delivered to lamellar bodies where it serves both to metabolise internalised and recycled surfactant PC and in modification of newly synthesised PC (Manevich and Fisher, 2005). Depletion of Prdx6 activity in Prdx6-/- mice results in a gradual accumulation of alveolar surfactant PC, decreased alveolar hydrolysis of [3H]DPPC-laden liposomes and decreased incorporation of [14C]-palmitate into DSPC (Fisher et al., 2005). Conversely, overexpression of Prdx6 in mice decreased alveolar and lung total PC and DSPC and increased both hydrolysis of alveolar DSPC and incorporation of [14C]-palmitate into DSPC (Fisher et al., 2006). Intriguingly, impaired trafficking of Prdx6 to lamellar bodies in ATII cells from the Pearl mouse, that lack the adaptor protein-3 complex, results in lamellar body phospholipidosis and increased half-life of alveolar surfactant PC (Kook et al., 2016). This observation is significant as the Pearl mouse is a model for the Hermansky Pudlak syndromes, a family of genetic disorders that result in lung fibrosis that is commonly lethal. Finally, a recent report suggest that Prdx6 also has lysophosphatidylcholine acyltransferase activity (LPCAT) that is relatively specific for incorporation of palmitoyl CoA into DSPC (Fisher et al., 2016).

An alternative LPCAT enzyme (LPCAT1) has been cloned from mouse and human lung (Chen et al., 2006; Harayama et al., 2009; Nakanishi et al., 2006) with a preference for incorporation of saturated acyl CoAs onto lysoPC. Partial embryonal deletion of LPCAT1 in mice caused decreased alveolar DSPC, which correlated with a variable expression of the enzyme and neonatal survival (Bridges et al., 2010). By contrast, LPCAT1-deficiency in mice lacking exon 3 did not result in neonatal lethality (Harayama et al.), but instead adult LPCAT1-deficient mice exhibited increased surface-tension lowering properties and poor lung compliance. While total phospholipid concentration in BALF was unchanged, the content of DPPC was almost halved with compensatory increased concentration of PC species containing polyunsaturated fatty acids.

Other gene defects in mice also impair surfactant function associated with altered molecular specificity of surfactant PC composition. For instance, lung compliance and surface tension-lowering properties of surfactant from BALF of long chain acyl CoA-deficient mice (LCAD-/-) are both significantly impaired, with decreased fractional content of DPPC (Goetzman et al., 2014). The mechanism underlying these effects are not yet clear, but LCAD is highly expressed in ATII cells and is responsible for fatty acid oxidation of long chain acylCoAs such as palmitoylCoA and OleoylCoA. One possible contributory factor is the reported accumulation of long chain acyl carnitines, the immediate substrate for LCAD, in alveolar fluid due to defective LCAD activity (Otsubo et al., 2015). Acyl carnitines co-isolate with surfactant and directly impair surfactant function.

1. **Neutral lipid and surfactant phospholipid metabolism**

Human lung surfactant contains approximately 10% cholesterol together with lower amounts of other neutral lipid such as triacylglycerol. Biophysical studies suggest cholesterol has a fluidising effect on surfactant surface activity (Nahak et al., 2014), the importance of which has been demonstrated by extensive comparative physiology studies. I contrast to the human lung, non-septal lungs of reptiles and amphibia contain much higher concentrations of surfactant cholesterol in both absolute and fractional amounts(Orgeig and Daniels, 2001). Moreover, hereothermic animals such as the fat tailed dunnart (Sminthopsis crassicauda) compensate for the effects on surfactant of decreased body temperature during topor by rapid variation of their lung surfactant concentration (Lopatko et al., 1999). Interestingly, this marsupial has surfactant with a predominantly monounsaturated not desaturated PC species composition, which has superior surface tension lowering properties at 23oC at the expense of poorer function at 37oC (Lang et al., 2005). Labelling studies indicate that >90% of mouse surfactant cholesterol is provided by direct transfer from plasma, probably derived from very low density lipoprotein (Guthmann et al., 1997). In contrast, cholesterol newly synthesised in the ATII cell is directed to the limiting membrane of the lamellar body and not secreted on lamellar body exocytosis . All available evidence suggests that cholesterol and phospholipid in surfactant are independently regulated and secreted (Orgeig and Daniels, 2001) and there is little evidence that cholesterol exerts a direct regulatory action on surfactant phospholipid metabolism. For instance, while loading ATII cells with β-cyclodextrin-cholesterol increased incorporation of 14C-palmitate into PC, this increment was modest compared with the much greater increase in cholesterol esterification, suggesting a compensatory role of PC in lipid droplet formation (Kolleck et al., 2002). By contrast, pharmacological depletion of serum cholesterol decreased disaturated PC in lamellar bodies but had no effect on lamellar body or secreted surfactant cholesterol concentration (Davidson et al., 1997). Additionally, transgenic mouse studies clearly distinguish between the regulations of phospholipid and neutral lipid metabolism by ATII cells. For instance, targeting Insig deletion to ATII cells dramatically increased cholesterol ester and triacylglycerol concentrations of both whole lung tissue and BALF, with accumulation of neutral lipid enriched lipid droplets in ATII cells (Plantier et al., 2012). Importantly, however, this manipulation had no effect on either the concentration or synthetic rate of surfactant PC.

1. **Stable isotope analysis of surfactant phospholipid synthesis**

Stable isotopes have been used to probe lipid biosynthesis ever since the first application of deuteriated heavy water (2H2O) to monitor metabolism in the mid-1930s (Schoenheimer and Rittenberg, 1936) and the use of isotope ratio mass spectrometry or gas chromatography are well-established techniques to monitor de novo lipogenesis (DNL). However, as the end products of DNL are the fatty acids palmitate and its chain elongation and desaturase products, it cannot provide insights into the metabolism of unsaturated fatty acids that must be provided from the diet. Moreover, this analytical approach requires hydrolysis of lipid species followed by subsequent derivatisation, typically as fatty acid methyl esters. Consequently, DNL analysis also cannot be used to probe any of the acyl remodelling mechanisms outlined above, for which analysis of individual molecular species of PC is essential.

Cogo and her colleagues have applied the DNL analytical approach to study incorporations *in vivo* of 13C-fatty acids (Cogo et al., 1999), 13C-glucose or deuterated water (Cogo et al., 2005) into palmitate of surfactant PC of preterm infants with neonatal respiratory distress syndrome (nRDS) (Verlato et al., 2008), term infants with congenital diaphragmatic hernia CDH (Cogo et al., 2002), and in adult patients ventilated for Acute Respiratory Distress Syndrome (ARDS) (Cogo et al., 2007; Simonato et al., 2011). These studies have shown a very long DSPC half-life and synthesis in preterm infants with nRDS, an unaltered fractional synthetic rate (FSR) of DSPC synthesis in infants with CDH and an increased FSR in ARDS, associated with a significantly lower alveolar DSPC pool size. Analysis of DPPC kinetics in CDH, monitored by intra-tracheal administration of 13C-DPPC, showed decreased half-life and smaller alveolar pool size

Tandem ESI-MS/MS offers an alternative approach for the analysis of lung surfactant PC metabolism *in vivo*. It permits analysis of incorporation of stable isotope-labelled substrates into PC molecular species, which in turn can determine both the molecular specificity of PC synthesis *de novo* and the extent and specificity of acyl remodelling mechanisms. Additionally, tandem ESI-MS/MS is extremely sensitive, very rapid and requires minimal sample preparation. In principle, tandem ESI-MS/MS can monitor the incorporation of a wide range of labelled substrates, but currently incorporation of *methyl*-9-[2H]-choline chloride has been most widely used for characterisation of PC synthesis (Postle et al., 2007). As choline is the common headgroup for all PC and sphingomyelin molecular species, incorporation of labelled choline can then monitor the metabolism of all their individual molecular species, giving estimates of synthesis, turnover and acyl remodelling. The sensitivity for determination of enrichments of *methyl*-9-[2H]-choline incorporated into PC species comes from the configuration of the triple quadrupole tandem mass spectrometer, which permits scanning for a class of phospholipid. Fragmentation of all PC molecules within the mass spectrometer generates a phosphoryl headgroup fragment of mass:charge (m:z) 184; precursor scans of m/z 184 (P184) is commonly used to reconstruct the original PC spectrum to give a diagnostic scan. The *methyl*-9-[2H]-choline labelled phosphorylcholine headgroup, at m:z 193, is similarly diagnostic for newly synthesised PC, and a precursor scan of m:x 193 (P193) then can reconstruct the spectrum of all labelled PC and sphingomyelin species. Comparison of the signal intensities between ions in the P184 scan and the equivalent ions nine mass units higher in the P193 scan, then provides a direct measure of enrichment of labelled choline in all PC species. This information can then be used to calculate fractional synthetic rates, turnover rates, half-lives and acyl remodelling.

This ESI-MS/MS approach was first applied to quantify surfactant synthesis in human volunteers *in vivo* by measurement of incorporation of labelled choline into PC isolated from induced sputum (Bernhard et al., 2004). *Methyl*-9-[2H]-choline was administered by a three hour i.v. infusion and resulted in maximal incorporation between 20 and 48 hours, with a slow subsequent decline. Importantly, the fractional incorporation of label into DPPC was much lower than the fractional concentration of unlabelled DPPC, but increased with time to reach an equilibrium by 24 hours. This initial study was followed up by a comparison of incorporation of *methyl*-9-[2H]-choline into PC of both BALF and sputum from human volunteers (Dushianthan et al., 2014b). Bronchoscopy was only performed at 24 and 48 hours, and the composition of labelled PC was at equilibrium with unlabelled PC by 24, in contrast to induced sputum where, again, equilibrium was only reached by 48 hours. This result was interpreted as a reflection of the time taken for PC, newly synthesised by the ATII cell, to travel from the alveolus to the higher bronchial tree, the presumed site of induced sputum generation. These analyses in volunteers support comparable analysis in control and surfactant protein D-/- mice, in which newly synthesised PC in BALF 1.5 hours after injection i.p. of *methyl*-9-[2H]-choline was relatively enriched in unsaturated species (Pynn et al., 2011). Equilibrium distributions of unlabelled and labelled PC were then achieved by 3 hours. Finally, analysis of small volume BALF samples from adult patients with ARDS showed considerable alterations to PC concentration, composition and enrichment of *methyl*-9-[2H]-choline into PC species (Dushianthan et al., 2014a). BALF samples taken from ARDS patients soon after intubation had significantly impaired concentration and composition of PC compared with healthy controls. Total BALF PC concentration was only 30% of control values in ARDS patients and the fractional concentration of DPPC of this lower amount of total PC was 50% that of controls. Incorporation of *methyl*-9-[2H]-choline into BALF PC showed intriguing results over the subsequent duration of ventilation. *Methyl*-9-[2H]-PC enrichment was significantly enhanced at all time points in ARDS patients compared with the control group, but with a very wide distribution between individual patients. As with the control group, however, incorporation of labelled choline into DPPC was delayed compared with that into unsaturated PC species. These results suggest that multiple factors impact on lung surfactant PC metabolism in ARDS. The increased content of unsaturated PC species in BALF is possibly due to membrane fragments generated from the increased numbers of airway inflammatory cells in ARDS, while the labelling results suggest that the decreased number of ATII cells in ARDS that fail to maintain adequate synthesis and secretion of surfactant.

This analysis of specificity of newly synthesised surfactant PC has important implications for models of surfactant lipid sorting, storage and secretion. The results are not compatible with a linear model, with selection of newly synthesised lipid species into immature lamellar bodies, followed by processes of acyl remodelling to generate lamellar bodies with the appropriate surfactant composition and, finally, secretion of mature lamellar bodies into the alveolus. For such a mechanism, newly synthesised and endogenous surfactant would always have identical compositions, which is not the case. Instead, the results suggest processes of rapid exchanges between all lamellar bodies in ATII cells so that the lamellar bodies about to be secreted into the alveolus contain an equivalent proportion of newly synthesised PC.

1. **Future perspectives**

The design of mass spectrometry instrumentation is undergoing a rapid phase of development, with advances both in sensitivity and in the ability to characterise molecular structures in ever-greater detail. As with all techniques, the chosen platform will inevitably be a compromise between different considerations. For instance, the tandem MS/MS approach outlined above analyses purely on mass, without any prior chromatography. This has the advantage of measuring all metabolites under identical ionization conditions, which is essential for comparing scans for labelled and unlabelled PC. However, this will not distinguish between isomeric lipids of different structure but the same mass. Such information can readily be achieved by adding a chromatography step before the mass spectrometry, but at the expense of increasing the complexity of quantification due both to different ionisation conditions during gradient chromatography and to a varied response with variable co-elution of different species.

One significant recent advance has been the transition of imaging mass spectrometry from being a purely research tool in its own right to be more widely available on commercial mass spectrometry instrumentation. Imaging mass spectrometry typically uses a focused laser to raster across a tissue section, with the extracted plume of molecules at each pixel being analysed by mass spectrometry. This technique is ideally suited to imaging phospholipid distributions and is beginning to address one of the long-term limitations of lipid biology. While protein distributions can readily be visualised by a variety of approaches, such as antibody tagging or location of GFP-constructs, such approaches are not feasible for lipids and, consequently, their spatial distribution has traditionally only been determined using lipophilic dyes. Imaging mass spectrometry provides a means, for the first time, to determine the spatial distribution of lipids in a tissue. In the context of lung architecture, for instance, this approach as expected locates DPPC to the lung parenchyma but locates the arachidonoyl-containing PC species palmitoylarachidonoyl PC almost exclusively to the bronchiolar region (Murphy et al., 2008). One future advance will be to combine stable isotope labelling with mass spectrometry imaging to determine the location of lipid synthesis as well as lipid distribution. Instruments currently under development have sufficient resolution to distinguish between labelled and unlabelled lipids purely on accurate mass, but only with a minimum spatial resolution of about 30 µm. The ultimate goal will be to image lipid distributions and synthesis at the sub-cellular level, for instance in individual lamellar bodies, but unfortunately currently available mass spectrometers possess neither sufficient spatial nor mass resolutions to achieve this level of characterisation. Such a development, however, will be needed to address the uncertainties of lamellar body metabolism highlighted by stable isotope methodologies.

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**Legend to figure**

**Figure 1. Pathways of pulmonary surfactant PC lipid synthesis, recycling and catabolism.** Abbreviations: fatty acid synthase (FAS), Endoplasmic Reticulum (ER), Golgi apparatus (Go), lamellar bodies (LB), lipid droplet (LD).