Analysis of lung surfactant phosphatidylcholine metabolism in transgenic mice using stable isotopes

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Abstract
Stable isotope labelling of lipid precursors coupled with mass spectrometry-based lipidomic analyses and determination of isotope enrichment in substrate, intermediate and product pools provide the parameters needed to determine absolute flux rates through lipid pathways in vivo. Here, as an illustration of the power of such analyses we investigated lung phosphatidylcholine (PC) synthesis in Surfactant Protein-D (SP-D) null mice. These animals develop emphysema, foamy alveolar macrophages and an alveolar lipoproteinosis with increasing age. We used the incorporation of methyl-9-[³²H] choline chloride coupled with ESI-MS/MS to quantify absolute rates of lung surfactant PC synthesis and secretion in an SP-D⁻/⁻ mouse model, together with an analysis of the molecular specificity of lung PC synthesis. PC synthetic rates were comparable in control (0.52 µmoles/lung/h) and SP-D⁻/⁻ (0.69 µmoles/lung/h) mice, as were rates of surfactant PC secretion (29.8 and 30.6 nmoles/lung/h respectively). Increased lung PC in the SP-D⁻/⁻ mouse was due to impaired catabolism, with a rate of accumulation of 0.057 µmoles/lung/h. The relatively low rates of surfactant PC secretion compared with total lung PC synthesis were compatible with a suggested ABCA1-mediated basolateral lipid efflux from alveolar type II epithelial cells. Finally, PC molecular species analysis suggested that a proportion of newly-synthesised PC is secreted rapidly into the lung air spaces in both control and SP-D⁻/⁻ mice before significant PC acyl remodelling occurs.
1. Introduction

Use of stable isotope labelling of lipid precursors in conjunction with detailed, mass spectrometry-based, lipidomic analyses has yielded powerful methodologies for molecular level analysis of the specificity and dynamics of lipid synthesis in vivo and in vitro (Postle et al 2007, Postle and Hunt 2009). When combined with determination of isotope enrichment within substrate pools these data further permit calculation of absolute flux rates through pathways and even individual molecular species of lipids (Pynn et al 2011). Such tools, in addition to probing normal developmental, nutritional or pathology-based perturbations in lipid metabolism, can also provide greater insight into unexpected derangements/dysregulations of lipid metabolism that may accompany specific gene targeted modifications.

An illustrative example is provided by surfactant protein D (SP-D), a member of the collectin family of carbohydrate pattern recognition host defence proteins, synthesised and secreted by the lung alveolar epithelium (Persson et al 1988), but also by many other epithelial surfaces (Madsen et al 2000). In addition to its ability to opsonise and aggregate bacteria (Kuan et al 1992, Restrepo et al 1999) and to bind viral particles and fungi (Hartshorn et al 1994, Schelenz et al 1995), studies in a variety of SP-D null mice have highlighted an initially unexpected role for SP-D both in the maintenance of the integrity of alveolar structure and in the regulation of pulmonary surfactant phospholipid metabolism. SP-D−/− mice develop a progress emphysema-like destruction of alveolar septa with age characterised by a progressive alveolar lipoproteinosis and the accumulation of large foamy macrophages (Botas et al 1998, Korfhagen et al 1998). Incorporation [3H]palmitate and [14C]choline into SP-D−/− mouse lung tissue and bronchoalveolar lavage (BALF) disaturated phosphatidylcholine (Sat-
PC) has previously been described (Ikegami et al 2000), with increased labelling of lung tissue PC and accumulation of incorporated label in BALF PC up until 72h incubation. These results were interpreted as decreased PC catabolism in the SP-D\(^{-/}\) mouse compared with control mouse lungs.

Many studies have used Sat-PC analysis as a surrogate for dipalmitoylphosphatidylcholine (DPPC, PC16:0/16:0), the major surface-tension lowering component of lung surfactant; the fundamental importance of DPPC for optimal function of human lung surfactant was established soon after the identification of surfactant deficiency as the primary cause of respiratory distress syndrome in preterm infants (Avery and Mead 1959, Klaus et al 1961). SAT-PC has typically been analysed as the residue after oxidation of surfactant lipid extracts with OsO\(_4\) (Mason et al 1976). In this experimental approach, OsO\(_4\) complexes with double bonds in the unsaturated PC species leaving disaturated phospholipids as the chloroform-soluble residue. The OsO\(_4\) technique has the advantages of being rapid, amenable to analysis of relatively large numbers of samples and providing a simple result. However, SAT-PC analysis is not specific for DPPC, but also includes other PC species such as palmitoylmyristoyl PC (PC16:0/14:0) (Hunt et al 1991), which can in turn be a significant surfactant component, for instance in the suckling post-natal rat (Bernhard et al 2007). This example illustrates the importance of the distribution of individual phospholipid molecular species for studies of surfactant metabolism and function. We have previously demonstrated the usefulness of surfactant phospholipid molecular species molecular species analysis in a variety of respiratory diseases, including asthma (Wright et al 2000), cystic fibrosis (Postle et al 1999) and paediatric acute lung injury (Todd et al 2010).
Numerous studies have detailed the importance of acyl remodelling mechanisms in the synthesis of DPPC (Stymne and Stobart 1985, Batenburg et al 1978, Caesar et al 1991). Up to 50% of lung DPPC is not synthesised directly from diacylglycerol (DAG), but is remodelled from other PC species by the sequential actions of phospholipase and acyltransferase enzymes. These analyses have shown an initial synthesis of unsaturated PC, typically palmitoyllinoleoyl PC (PC16:0/18:2) or palmitoyloleoyl PC (PC16:0/18:1), their subsequent conversion to 1-palmitoyl lysoPC (LPC16:0) by the action of a phospholipase A2, followed finally by re-esterification to DPPC by the addition of palmitoyl-CoA, catalysed by lysoPC acyltransferase (LPCAT) (Okuyama et al 1983). Indeed, the critical importance of LPCAT for surfactant function has recently been highlighted by the demonstration that the null mutation is fatal in neonatal mice (Chen et al 2006).

One final factor in surfactant metabolism that has rarely been considered in detail is the temporal relationship between mechanisms of phospholipid synthesis and the molecular specificity of surfactant secretion from the type II cell. The assumption of studies which typically involve pre-labelling surfactant PC for 24 hours followed by hormonal stimulus of secretion is that these are linear processes. Their inherent implication is that surfactant PC is synthesised de novo, acyl remodelled and packaged into lamellar bodies to acquire its mature equilibrium composition before lamellar body contents are secreted into the alveolar lumen. This assumption has been challenged by the use of the stable isotope methyl-9-[2H] choline administered to human volunteers and subsequent analysis by electrospray ionisation mass spectrometry (ESI-MS) of methyl-9-[2H] incorporation into PC molecular species of sequential induced-sputum samples Bernhard et al 2004). This analysis showed a time-dependent redistribution of incorporated stable isotope label towards DPPC, strongly suggesting both that a proportion of newly-synthesised surfactant PC is secreted before acyl
remodelling is complete and that the processes of acyl remodelling and secretion must be independently regulated.

We have used the same ESI-MS methodology to investigate the mechanisms underlying the dysregulation of surfactant PC metabolism in an SP-D⁻/⁻ mouse model. Here we report the relative contributions of altered PC synthesis and turnover to the accumulation of alveolar phospholipids in the SP-D⁻/⁻ mouse and whether this phospholipid accumulation is accompanied by any alteration to the molecular specificity of surfactant PC synthesis or acyl remodelling.

2. Materials and methods

2.1 Animal labelling

Standard homologous techniques were used to generate mice deficient in SP-D as previously described. The deficient mice were imported from the laboratory of Professor Sam Hawgood, University of California, San Francisco. The SP-D knock-out mice, back-crossed more than 10 generations into a C57BL/6 background were fed ad libitum and housed in isolators in a pathogen free environment. Pathogen free C57BL/6 wild-type age and sex, matched controls were obtained from Harlan-OLAC (Shaw’s Farm, Bicester, Oxfordshire, UK). Eight week old female were kept in a normal light-dark cycle and had free access to pelleted food and water. Animals received appropriate human care according to criteria outlined in the NIH guidelines for the care and use of laboratory animals. Animals received 100μl intraperitoneal injections of methyl-9-[²H] choline chloride (10mg/ml solution in sterile physiological saline) and were sacrificed at 1.5, 3, 6 and 24 hours (n = 5 per time point/group) after labelling. Mice
were killed by carbon dioxide asphyxia, their tracheas rapidly canulated, lungs lavaged in situ four times with 0.9% (w/v) saline and the volume recorded. Recovered bronchoalveolar lavage fluid (BALF) was placed on ice and lung parenchyma was rapidly dissected away from main bronchi and flash frozen in liquid nitrogen. Frozen lung tissue was transferred to pre-weighed sample vials, lung weights determined and samples then stored at -80 °C until extraction. BALF was centrifuged at 400 x g x 10 min at 4 °C to pellet alveolar macrophages and supernatants stored at -80 °C until extraction.

2.2 ESI-MS analysis of phosphatidylcholine and choline phosphate

Frozen lavaged lungs were homogenised in 3 ml of 0.9% saline using an Ultra-Turrax homogeniser (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). Aliquots (800 μl) of the lung homogenates or BALF were lipid extracted with chloroform and methanol (Bligh and Dyer 1959) after adding dimyristoyl PC (PC14:0/14:0) as internal standard. Trace amounts of endogenous PC14:0/14:0 would contribute a maximum quantification error of 0.6% and 1.3% for lung tissue and BALF respectively, values within the experimental error of the analysis. The chloroform-rich lower phases were recovered, dried under nitrogen gas and stored at -20 °C for ESI-MS analysis. The upper phases from the tissue extracts were collected and lyophilised, and choline phosphate isolated according to the following method (Burge et al 1993). The residues were dissolved in 1ml 0.05% ammonia/methanol (1:9, v/v), sonicated for 2 minutes and centrifuged at 13,000 x g x 30 secs to remove particulates. The supernatants were applied to aminopropyl solid phase cartridges (Varian Inc. Palo Alto, USA) previously conditioned with 1 ml 0.05% ammonia/methanol (1:9, v/v). Loaded cartridges were then washed with 1ml (0.05% ammonia/methanol (1:9, v/v) and choline phosphate eluted with 1.5ml 0.05% ammonia: 6% (w/v) NH₄HCO₃: methanol (1:1:4, v/v).
The eluates were dried under nitrogen gas and re-suspended in methanol (100μl) for analysis by ESI-MS/MS.

PC species were quantified using a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshaw, UK) equipped with an electrospray ionisation interface. Samples were dissolved in methanol:chloroform:water: 25% NH₄OH (7:2:0.8:0.2, v/v) and introduced into the mass spectrometer by direct infusion. Collision gas-induced dissociation (CID) using argon gas produced protonated phosphocholine head group fragments with m/z = +184 corresponding to endogenous PC and m/z = +193 for PC synthesised via the CDP-choline pathway (Hunt et al 2001). Endogenous PC molecular species were quantified from diagnostic precursor ion scans of the m/z =184 fragment ion (P184) while newly synthesised PC was quantified from comparable diagnostic precursor ion scans of the m/z=193 fragment ion (P193). Endogenous and methyl-9-[^2]H labelled choline phosphate were quantified by ESI-MS/MS operated in the multiple reaction monitoring (MRM) mode, monitoring the transition from the precursor ions m/z = +183.8 and m/z = +192.8 to product fragment ions m/z = +85.8 and m/z = +94.8 respectively using a dwell time of 0.1 seconds.

PC molecular species and incorporation of methyl-9[^2]H choline were calculated using self-extending Excel macro programmes developed in house. MS spectra were smoothed, baseline-subtracted, converted to centroid format and exported to individual Excel sample files. These were imported into the analyser programme and corrected for the [M+1]⁺ and [M+2]⁺ ions generated by the approximately 1% natural abundance of ¹³C. The fractional incorporations of methyl-9[^2]H choline into individual PC molecular species were then calculated relative to the total abundance given by Σ(P184 + P193).
3. Results

3.1 Methyl-9-[^2H] choline incorporation into lung and BALF

Choline is incorporated into PC by the CDP:choline pathway, whereby it is converted first to choline phosphate and then CDP:choline by the sequential actions of choline kinase and CTP:cholinephosphate cytidylyltransferase (CCT). CDP:choline then complexes with DAG by the action of cholinephosphotransferase to form PC. The composition of newly synthesised PC will reflect that of the DAG substrate and, if the CDP:choline is labelled with deuterium atoms, these will be retained in the synthesised PC. ESI-MS can then readily distinguish between endogenous and newly-synthesised PC molecules by precursor scans of respectively \( m/z \) 184 (P184) and 193 (P193), these being the CID-generated fragment ions of choline phosphate and methyl-9-[^2H] choline phosphate. This is shown clearly in Figure 1, which details P184 and P193 scans of total lipid extracts from a lavaged lung (panels A and B) of a control mouse 1.5 hours after the i.p. injection of 1 mg methyl-9-[^2H] choline chloride. Both spectra have been normalised to the highest abundance ion peak displayed; the respective fractional incorporations are shown below in Figure 2. Importantly, although newly synthesised and endogenous PC had relatively similar compositions, these were not identical. For example, the proportion of PC16:0/18:2 and PC16:0/18:1 were higher in newly synthesised PC compared with endogenous PC, but there was negligible incorporation into the corresponding stearoyl species (PC18:0/18:2 and PC18:0/18:1).

The fractional incorporations of methyl-9-[^2H] choline incorporation into PC were calculated for the sum of the 19 molecular species that individually contributed greater than 1 mole % of total PC. This value was given by equation 1:
Fractional intensity = \( \frac{(\Sigma P_{193} \times 100)}{\Sigma P_{193} + \Sigma P_{184}} \) %, where \( \Sigma P_{193} \) and \( \Sigma P_{184} \) are sums of the respective ion intensities after correction for \(^{13}\)C isotope abundance and differential CID fragmentation. Lung fractional incorporation was maximal at 6h for control and at 3h for SP-D\(^{-}\)-mice and then declined by 24h for both groups (Fig 2A). At all time points, the extent of the fractional enrichment for the SP-D\(^{-}\)-mouse was consistently about half that of control mice (Fig 2A). Incorporation into BALF PC was measureable by 1.5h and then increased progressively until 24h; enrichment of BALF PC was again much lower in the SP-D\(^{-}\)-compared with control mice at all time points (Fig 2B). These comparisons are somewhat misleading, however, as total PC concentration in the lavaged SP-D\(^{-}\)-mouse lung was higher than in control mouse lung (2.20±0.69 vs 1.54±0.26 μmoles/lung, n=20, p<0.001), with corresponding values for BALF PC being 0.54±0.22 and 0.26±0.12 μmoles/lavage (p<0.001).

There was proportionally more PC recovered in the lavage fraction in the SP-D\(^{-}\)-mouse (19.95±5.64) than in the control mouse (13.07±3.48 % total lung PC, p<0.001). Taking these differences into account, rates of incorporation/lung of methyl-9-[\(^{2}\)H] choline were more comparable between the two groups.

3.2 Calculation of lung PC synthesis and turnover

The extent of incorporation of a labelled substrate is the product of the measured enrichment corrected for label enrichment in the substrate. Consequently, some of the incorporation discrepancy between SP-D\(^{-}\)- and control mice could have been due to a difference in substrate enrichment. This was indeed the case as enrichment of methyl-9-[\(^{2}\)H] choline phosphate (CP), the substrate for CCT, was consistently lower in lavaged lungs from SP-D\(^{-}\)-
compared with control mice (Fig 3). We assume that, although the SP-D−/− mice were fully 
back-crossed, a mouse strain difference with the control C56BL mice explained the lower 
enrichment of substrate methyl-9-[2H] choline phosphate. It was unlikely to be a direct 
consequence of absence of SP-D in the lungs as enrichment of methyl-9-[2H] choline 
phosphate was similarly lower at all time points in parallel analysis of mouse spleen from SP-
D−/− compared with control mice (results not shown).

Knowledge of fractional enrichments of PC and choline phosphate, lung weight and total 
lung PC concentration enabled us to estimate apparent absolute amounts of lung PC 
synthesised at each time point (t) from the equation 2:

\[
\text{PC synthesis}_t = \frac{\text{PC enrichment}_t}{\text{CP enrichment}_t} \times \frac{[\text{PC}]_t}{\text{weight}} \text{ μmoles/lung}
\]

Initial rates of lung PC synthesis were calculated over the first 3h after administration of 
methyl-9-[2H] choline chloride (Fig 4). More intermediate time points would have been 
required for an accurate correlation, but using the results at 1.5 and 3h suggested that lung PC 
synthesis was slightly faster in the SP-D−/− mouse (0.69 μmoles/lung/h) compared with control 
mice (0.52 μmoles/lung/h). They also suggested that analysis of a single time point at 3h 
would provide a reasonable assessment of the rate of lung PC synthesis for both groups of 
mice. The amount of PC synthesised by the control mouse lungs reached a plateau value of 
1.71±0.31 μmoles/lung between 6 and 24h, which was in close agreement with the analysed 
lung PC content (1.54±0.26 μmoles/lung, Fig 4, lower dotted horizontal line). This plateau
value represented the equilibrium between PC synthesis by the lungs and loss of PC from lung tissue by a combination of surfactant secretion, PC catabolism within the lungs and potentially from basolateral secretion of unsaturated species from type II pneumocytes into the circulation (Zhou et al 2004, Agassandian et al 2004). By definition, of course, these values in an equilibrium state must be identical.

The similar calculation for the SP-D−/− mouse gave a very different result, in that after the initial linear synthesis over the first 3h, the apparent rate of lung PC synthesis continued to increase up until 24h to a value greater than measured total lung PC content for this mouse (2.20±0.69 μmoles/lung, Fig 4, upper dotted horizontal line). In reality, of course, it is not possible for the lungs to contain more synthesised PC than total PC content, and this discrepancy was a direct reflection of decreased lung PC catabolism in the SP-D−/− mouse coupled with the progressive decline with time of isotope enrichment in the choline phosphate substrate pool. Increased residence of PC synthesised at earlier incubation time points, when corrected for the lower choline phosphate enrichment at the later times, would lead to this apparent anomaly. More time points would be required for an accurate assessment from this analysis of the rate of impaired lung PC catabolism in the SP-D−/− mouse, but an upper value of 0.057 μmoles/lung/h can be estimated from the results between 6 and 24h as a measure of the rate of accumulation of lung PC. This is almost certainly an over-estimate, but illustrates how a relatively small imbalance between synthesis and catabolism is sufficient to account for the slow accumulation of PC in the SP-D−/− mouse lung.

3.3 PC secretion and turnover in BALF
Newly synthesised PC (% total methyl-9-[\textsuperscript{2}H] choline incorporation) was detectable in BALF at 1.5h (Fig 5); extrapolation of the data suggested that the lag time for secretion of newly synthesised surfactant PC was just under one hour (mean 0.94h). Initial accumulation of label between 1.5 and 6h was slightly greater for SP-D\textsuperscript{+} mice (1.47 % total incorporation/h) than for control mice (1.25 % total incorporation/h), but this difference was not significant until 24h (Fig 5). These proportions of secreted PC at 24h were comparable to the distributions of endogenous PC in BALF. Correcting this percentage secretion for substrate choline phosphate enrichment, the apparent rates of PC secretion for the first 6h after label administration were identical for SP-D\textsuperscript{+} mice (30.6 nmoles/h) and control mice (29.8 nmoles/h) (Fig 6).

### 3.4 Specificity of surfactant phosphatidylcholine synthesis

In addition to estimates of total PC flux, ESI-MS facilitated the analysis of the molecular specificity of acyl remodelling in surfactant PC synthesis and secretion. Figure 7 shows the relative incorporations of methyl-9-[\textsuperscript{2}H] choline into BALF PC molecular species. Species characteristic of lung surfactant (PC16:0/16:0 and PC16:0/16:1) were relatively under represented in newly secreted PC at 1.5 and 3h, and then increased to equilibrium endogenous values by 24h. By contrast, PC species more characteristic of cell membrane (expressed in figure 7 as the sum of the unsaturated species PC16:0/18:1, PC16:0/18:2, PC18:0/18:1 and PC16:0/20:4) were relatively enriched in newly secreted BALF PC and declined progressively with time. This analysis not only demonstrated that acyl remodelling mechanisms were active in lung PC synthesis, and were essentially identical for SP-D\textsuperscript{+} and control mice, but that the processes of acyl remodelling, lamellar body assembly and secretion do not form a linear sequence. If these processes were linear, implying that all
newly-synthesised surfactant PC had to achieve the mature composition before secretion, then all newly synthesised and secreted surfactant PC would have been expected to display the same composition at all time points after label administration, and this was clearly not the case.

4. Discussion

Total lung phospholipid content of the SP-D−/− mouse has been reported to increase progressively compared with control mice up until at least 18 weeks (Botas et al 1998) after birth. As we wished to characterise the mechanisms responsible for this phenomenon, we chose to study mice at 8 weeks of age when the lung lipid accumulation in the SP-D−/− mouse is first apparent. The incorporation results presented in Figs 2, 3 and 4 represent a novel approach to the analysis of synthesis and turnover of lung PC. Correction of lung PC methyl-9-[2H] enrichment (Fig 2) both for lung PC concentration and for choline phosphate enrichment demonstrated a the linear rate of PC synthesis calculated over the first 3h which was slightly higher for SP-D−/− compared with control mice (Fig 4). For control mice, which regulate a constant lung PC concentration, the rate of PC catabolism and/or export must equal this rate of synthesis, giving a turnover time of 3.19h calculated by dividing lung PC concentration by synthetic rate. One consequence of this conclusion is that, for analyses at 6h and 24h, all lung PC was effectively synthesised in the proceeding 3h period. This interpretation is supported by the observation that the total amount of newly synthesised PC (μmole/lung) reached a plateau from 3-24h that was very close to the lung PC concentration calculated from the P184 scan. In contrast, comparable analysis for the SP-D−/− mouse demonstrated that the rates of lung PC synthesis and loss were not at equilibrium (Fig 4).
These calculations also provide novel insights into the relative dynamics of PC turnover in whole lung and in lung surfactant. The rate of PC turnover in BALF in control mice was 9 nmoles/h, while the rate of lung PC turnover was 57 nmoles/h. This observation shows clearly that metabolism of secreted alveolar surfactant makes a relatively minor contribution to the overall loss of newly synthesised PC from the lungs. It is unlikely that phospholipase A$_2$-mediated hydrolysis, for instance, is a major contributor to mouse lung PC catabolism, as concentration and incorporation of methyl-9-[${}^3$H] choline into lung lysoPC remained low throughout the 24h labelling period (results not shown). The most likely mechanism responsible for loss of newly synthesised PC from the lung is export of PC from the type II pneumocyte into the circulation and/or lymph fluid. This has been reported to be mediated by ABCA1, with ApoA1 as the acceptor, is stimulated to compensate for increased lung PC synthesis (Zhou et al 2004) and is relatively specific for longer acyl chain unsaturated PC species. The importance of shorter acyl chain length for selection of PC species to be packaged into lamellar bodies and secreted surfactant (Postle et al 2006) is shown clearly by the increased fractional synthesis of PC16:0/16:1 with time. The significance of this mechanism for removal of PC and cholesterol from lung is shown clearly by the elevated lung lipid content and respiratory distress experienced by ABCA1 deficient mice (Bates et al 2005).

Finally, the pattern of surfactant PC synthesis and secretion into BALF also has important implications for understanding of mechanisms regulating the molecular specificity of surfactant PC composition. The importance of acyl remodelling mechanisms in the synthesis of surfactant PC16:0/16:0 has long been appreciated (Okuyama et al 1983), and redistribution with time of the incorporated methyl-9-[${}^3$H] choline headgroup from an initially synthesised unsaturated PC towards PC16:0/16:0 was indeed observed as expected in both SP-D$^+$ and
control mice (results not shown). The novel and significant observation here (Fig 7) was that a similar redistribution of headgroup methyl-9-[\(^2\)H] choline label was also observed for both groups of mice. This demonstrates that newly-synthesised PC is rapidly packaged into lamellar bodies and secreted into the alveolus before acyl remodelling processes are complete, in agreement with our previous analysis of the specificity of methyl-9-[\(^2\)H] choline incorporation into induced sputum PC in adult human volunteers (Bernhard et al 2004). The fundamental implication from this observation is that assembly of PC in lamellar bodies followed by secretion is not a completely linear process.

The rapid synthesis and turnover of total PC in mouse lung is characteristic of a biological system with a requirement to respond rapidly to altered physiological conditions. As with enzyme protein turnover (Walker 1983), rapid degradation with a high turnover is a pre-requisite for the lungs to be able to increase surfactant PC synthesis rapidly in response, for instance, to alveolar stretch (Torday and Rehan 2002). Moreover, this analysis suggests that this mechanism for bulk lung PC turnover is not significantly altered in the SP-D\(^{-}\) mouse, supporting previous conclusions that the accumulation of lung and alveolar PC in the SP-D\(^{-}\) is a consequence of impaired catabolism. Studies of the catabolism of tracer amounts of \(^3\)H]PC16:0/16:0 administered together with exogenous surfactant strongly suggest that, unlike in the granulocyte-macrophage colony-stimulating factor deficient mouse that also develops an alveolar lipidosis, uptake and degradation of surfactant PC by alveolar macrophages is not deficient in the SP-D\(^{-}\) mouse (Ikegami et al 2000). Consequently, uptake and processing of surfactant PC as part of alveolar surfactant recycling are the most likely causes of the specific PC catabolism defect in the SP-D\(^{-}\) mouse.
Legends to Figures

Figure 1. Electrospray ionisation mass spectrometric analysis of endogenous and newly synthesised mouse lung phosphatidylcholine. A control mouse was injected i.p. with 1mg methyl-9-[\(^2\)H] choline chloride and killed 1.5h later. Lungs were lavaged, homogenised and extracted as described in the methods section. Endogenous PC was selectively detected by diagnostic precursor scan of the fragment ion \(m/z\) 184\(^+\) (panel A) and incorporated stable isotope by precursor scan of \(m/z\) 193\(^+\) (panel B). Each spectrum is normalised to PC16:0/16:0, the most abundant species on display. PC species with the same fatty acid distributions are 9 amu higher in the newly synthesised 9-[\(^2\)H]PC. Spectral data for Panel B was collected for x3 that for Panel A.

Figure 2. Incorporation of methyl-9-[2H] choline into mouse lung and bronchoalveolar lavage phosphatidylcholine in vivo. SP-D\(^-\) mice (squares) and C57BL6 control mice (circles) were all injected with 1mg methyl-9-[\(^2\)H] choline chloride and sacrificed at the indicated times. Total lipid was extracted from lavaged lungs (panel A) and bronchoalveolar lavage fluid (panel B) and analysed by ESI-MS/MS as P184\(^+\) and P193\(^+\) scans. Fractional enrichment of the methyl-9-[\(^2\)H] choline-labelled PC was determined after correction for \(^{13}\)C and differential fragmentation as \% total PC in the extract. Data is presented as mean±S.D., n=3-5.

Figure 3. Fractional enrichment of choline phosphate from mouse lung. Choline phosphate was isolated from the aqueous fraction of the lipid extracts of mouse lung
homogenate shown in figure 2 and analysed by MRM monitoring of the 183.8→85.8 transition for endogenous and the 192.8→94.8 transition for stable isotope labelled choline phosphate. Data is presented as mean±S.D., n=3-5, * p<0.01.

**Figure 4. Synthesis of phosphatidylcholine by mouse lung in vivo.** The enrichment data presented in figure 2 was corrected for total PC content and the fractional enrichment of methyl-9-[2H] choline phosphate of lavaged lung from SP-D+/− mice (squares) and C57BL6 control mice (circles) to give an estimate of the apparent concentration of newly synthesised PC in lavaged lung (μmole/lung, mean±S.D., n=3-5). Rates of synthesis were calculated from the data at 1.5h and 3h (dashed lines). Total mean lung PC is shown by the horizontal dotted lines.

**Figure 5. Secretion of newly synthesised phosphatidylcholine into bronchoalveolar lavage fluid.** Appearance of methyl-9-[2H] choline label in BALF PC was calculated as a percentage of the sum of incorporation of the stable isotope into lavaged lung and BALF for SP-D+/− mice (squares) and C57BL6 control mice (circles). The proportion of total endogenous lung PC recovered in BALF is shown for comparison. Results are presented as mean±S.D., n=3-5, * p<0.05.

**Figure 6. Phosphatidylcholine secretion by mouse lung.** The apparent concentration of newly synthesised PC recovered in BALF was calculated as nmoles/total recovered lavage fluid. Total mean BALF PC is shown by the horizontal dotted lines. Results are presented as mean±S.D., n=3-5, * p<0.05.
Figure 7. Distribution of incorporated methyl-9-[3H] choline label in newly synthesised phosphatidylcholine molecular species from bronchoalveolar lavage fluid. Results are presented for PC species characteristic of lung surfactant (circles, PC16:0/16:0; squares, PC16:0/16:1) and for the sum of PC species more characteristic of cell membranes (triangles, ΣPC16:0/18:1, PC16:0/18:2, PC18:0/18:1, PC16:0/20:4). Data was calculated as a percentage of total incorporation into identified PC species, mean±S.D., n=3-5.

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Figure 1

A  
Precursors of 184ES+
Integrity 7.30e7 cps

B  
Precursors of 193ES+
Integrity 3.72e6 cps
Figure 2

A

Fractional enrichment (% total PC)

0 6 12 18 24

0 1 2 3 4

B

- C57BL6 control mice
- SP-D:\- mice

Time (h)

0 6 12 18 24
Figure 3

![Graph showing fractional enrichment (%) over time (h) for C57BL6 control mice and SP-D−/− mice.](Image)

- C57BL6 control mice
- SP-D−/− mice

* , P<0.01
Figure 4

PC synthesis ($\mu$mole/lung) in SP-D−/− mice compared to C57BL6 control mice. The data indicates a significant difference (*, P<0.05) in PC synthesis between the two groups. The graph shows that the PC concentration increases with time, with the SP-D−/− mice having a higher PC concentration compared to the control group. The regression lines y=0.69x and y=0.52x are shown, indicating different rates of PC synthesis in the two groups.
Figure 5

Graph showing the BALF PC (% total incorporation) over time (h) for SP-D−/− mice and C57BL6 control mice. The endogenous PC in lavage is indicated by an asterisk (*), p=0.05.
Figure 6

The graph shows the synthesis of BALF PC (nmoles) over time (h) for two groups: SP-D−/− mice and C57BL6 control mice. The y-axis represents synthesis BALF PC (nmoles) ranging from 0 to 800, and the x-axis represents time (h) ranging from 0 to 24.

- SP-D−/− mice: represented by closed square markers.
- C57BL6 control mice: represented by closed circle markers.

A star (*) indicates p<0.05, suggesting a significant difference between the two groups at certain time points.
Figure 7

**SP-D⁻/⁻ mice**

**C57BL6 control mice**

Methyl-D₉ distribution (%) over time (h) for PC16:0/16:0, PC16:0/16:1, and sum unsaturated PC.
 We investigate lung PC accumulation in SP-D null mice with a stable isotope label
 Lipidomic analyses can show absolute rates of PC synthesis and secretion
 PC accumulation in SP-D null lungs is due to impaired lipid catabolism
 Excess lung PC synthesis is consistent with ABCA1-mediated basolateral lipid efflux
 A proportion of newly synthesised PC is rapidly secreted without remodelling
Model of phosphatidylcholine synthesis and turnover in mouse alveolar type II cells

Graphical Abstract

PC synthesis, alveolar secretion, turnover via macrophage (x) or type II cell (29.8-x) and extrapulmonary transport must balance at equilibrium. Since PC synthesis is >10 x alveolar turnover (520 nmoles/lung/h vs 29.8 nmoles/lung/h), export of excess PC is essential. Observed PC saturation may result in part from selective export of unsaturated PC. Implicit in this model is a recognition that lung is a significant extra-hepatic source of circulating, PUFA-enriched PC.