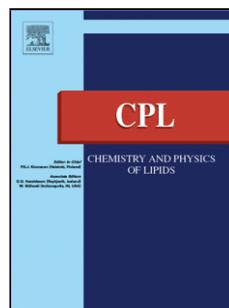


Accepted Manuscript

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

Authors: Anthony D. Postle, Neil G. Henderson, Griehof Koster, Howard W. Clark, Alan N. Hunt



PII: S0009-3084(11)00045-4

DOI: doi:10.1016/j.chemphyslip.2011.04.004

Reference: CPL 3996

To appear in: *Chemistry and Physics of Lipids*

Received date: 14-2-2011

Revised date: 9-4-2011

Accepted date: 10-4-2011

Please cite this article as: Postle, A.D., Henderson, N.G., Koster, G., Clark, H.W., Hunt, A.N., Analysis of lung surfactant phosphatidylcholine metabolism in transgenic mice using stable isotopes, *Chemistry and Physics of Lipids* (2010), doi:10.1016/j.chemphyslip.2011.04.004

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Anthony D. Postle, Neil G. Henderson, Grielof Koster, Howard W. Clark and Alan N. Hunt

Division of Infection, Inflammation and Immunity

Faculty of Medicine, University of Southampton

Southampton, UK

Address for correspondence:

Alan Hunt, PhD

Sir Henry Wellcome Laboratories,

MP803, Level F, South Block

Southampton General Hospital

Tremona Road

Southampton

SO16 6YD

Tel: ++44(0)2380 794178

e-mail: anh@soton.ac.uk

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-[²H] 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 progressive 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 OsO₄ (Mason et al 1976). In this experimental approach, OsO₄ complexes with double bonds in the unsaturated PC species leaving disaturated phospholipids as the chloroform-soluble residue. The OsO₄ 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 palmitoylinoleoyl 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 (100 μ l) 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-[²H]* 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-[²H]* choline were calculated using self-extracting 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-[²H]* choline into individual PC molecular species were then calculated relative to the total abundance given by $\Sigma(P184 + P193)$.

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:cholinophosphate cytidylyltransferase (CCT). CDP:choline then complexes with DAG by the action of cholinophotransferase 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 = $\frac{(\Sigma P193 \times 100)}{(\Sigma P193 + \Sigma P184)}$ %, where $\Sigma P193$ and $\Sigma P184$ 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 $\mu\text{moles/lung}$, $n=20$, $p<0.001$), with corresponding values for BALF PC being 0.54 ± 0.22 and 0.26 ± 0.12 $\mu\text{moles/lavage}$ ($p<0.001$). 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-[^2H] 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-[^2H] 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:

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

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 ($2.20 \pm 0.69 \mu\text{moles/lung}$, 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 $0.057 \mu\text{moles/lung/h}$ 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-[²H] 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^{-/-} 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^{-/-} 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-[²H] 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^{-/-} 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 ($\mu\text{mole/lung}$) 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 A₂-mediated hydrolysis, for instance, is a major contributor to mouse lung PC catabolism, as concentration and incorporation of *methyl-9-[²H] 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-[²H] 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-[²H] 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-[²H] 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 prerequisite 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 [³H]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 lipidoses, 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-[²H] 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-[²H]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-[²H] 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-[²H] choline*-labelled PC was determined after correction for ¹³C 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 ($\mu\text{mole/lung}$, 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-[²H] 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, Σ PC16:0/18:1, 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 \pm S.D., n=3-5.

References

- Agassandian, M., Mathur, S.N., Zhou, J., Field, F.J., Mallampalli, R.K. 2004, Oxysterols trigger ABCA1-mediated basolateral surfactant efflux. Am. J. Respir. Cell Mol. Biol. 31, 227-233
- Avery, M.E., Mead, J. 1959, Surface properties in relation to atelectasis and hyaline membrane disease. Am. J. Dis. Child. 97, 517-523
- Batenburg, J.J., Longmore, W.J., Van Golde, L.M.G. 1978, The synthesis of phosphatidylcholine by adult rat lung alveolar type 2 epithelial cells in primary culture. Biochim. Biophys. Acta 529, 160-170

Bates, S.R., Tao, J.Q., Collins, H.L., Francone, O.L., Rothblat, G.H. 2005, Pulmonary abnormalities due to ABCA1 deficiency in mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 289, L980-L989

Bernhard, W., Pynn, C.J., Jaworski, A., Rau, G.A., Hohlfeld, J.M., Freihorst, J., Poets, C.F., Stoll, D., Postle, A.D. 2004, Mass spectrometric analysis of surfactant metabolism in human volunteers using deuteriated choline. Am. J. Respir. Crit. Care Med. 2004, 170, 54-58

Bernhard, W., Schmiedl, A., Koster, G., Orgeig, S., Acevedo, C., Poets, C.F., Postle, A.D. 2007, Developmental changes in rat surfactant lipidomics in the context of species variability. Pediatr. Pulmonol. 42, 794-804

Bligh, E.G., Dyer, W.J. 1959, A rapid and sensitive method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917

Botas, C., Poulain, F., Akiyama, J., Brown, C., Allen, L., Goerke, J., Clements, J., Carlson, E., Gillespie, A.M., Epstein, C., Hawgood, S. 1998, Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. Proc. Natl. Acad. Sci. U S A 95, 11869-11874

Burdge, G.C., Kelly, F.J., Postle, A.D. 1993, Synthesis of phosphatidylcholine in guinea-pig fetal lung involves acyl remodelling and differential turnover of individual molecular species. *Biochim. Biophys. Acta* 1166, 251-257

Caesar, P.A., McElroy, M.C., Kelly, F.J., Normand, I.C.S., Postle, A.D. 1991, Mechanisms of phosphatidylcholine acyl remodelling by human fetal lung. *Am. J. Respir. Cell. Mol. Biol.* 5, 363-370

Chen, X., Hyatt, B.A., Mucenski, M.L., Mason, R.J., Shannon, J.M. 2006, Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells. *Proc. Natl. Acad. Sci. U S A* 103, 11724-11729

Hartshorn, K.L., Crouch, E.C., White, M.R., Eggleton, P., Tauber, A.I., Chang, D., Sastry, K. 1994, Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *J. Clin. Invest.* 94, 311-319

Hunt, A.N., Kelly, F.J., Postle, A.D. 1991, Developmental variation in whole human lung phosphatidylcholine molecular species. *Early Human Devel.* 25, 156-171

Hunt, A.N., Clark, G.T., Attard, G.S., Postle, A.D. 2001, Highly saturated endonuclear phosphatidylcholine is synthesized in situ and colocated with CDP-choline pathway enzymes. *J. Biol. Chem.* 276, 8492-8499

Ikegami, M., Whitsett, J.A., Jobe, A., Ross, G., Fisher, J., Korfhagen, T. 2000, Surfactant metabolism in SP-D gene-targeted mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 279, L468-L476

Ikegami, M., Hull, W.M., Yoshida, M., Wert, S.E., Whitsett, J.A. 2001, SP-D and GM-CSF regulate surfactant homeostasis via distinct mechanisms. Am. J. Physiol. Lung Cell Mol. Physiol. 281, L697-L703

Klaus, M.H., Clements, J.A., Havel, R.J. 1961, Composition of surface-active material isolated from beef lung. Proc. Natl. Acad. Sci. U S A 47, 1858-1859

Korfhagen, T.R., Sheftelyevich, V., Burhans, M.S., Bruno, M.D., Ross, G.F., Wert, S.E., Stahlman, M.T., Jobe, A.H., Ikegami, M., Whitsett, J.A., Fisher, J.H. 1998, Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. J. Biol. Chem. 273, 28438-28443

Kuan, S.F., Rust, K., Crouch, E. 1992, Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an Escherichia coli-binding protein in bronchoalveolar lavage. J. Clin. Invest. 90, 97–106

Madsen, J., Kliem, A., Tornoe, I., Skjodt, K., Koch, C., Holmskov, U. 2000, Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J. Immunol.* 164, 5866-5870

Mason, R.J., Nellenbogen, J., Clements, J.A. 1976, Isolation of disaturated phosphatidylcholine with osmium tetroxide. *J. Lipid Res.* 17, 281-284

Okuyama, H., Yamada, K., Miyagana, T., Suzuki, M., Prasad, R., Lands, W.E.M. 1983, Enzymatic basis for the formation of pulmonary surfactant lipids by acyltransferase systems. *Arch. Biochem. Biophys.* 221, 99-107

Persson, A., Rust, K., Chang, D., Moxley, M., Longmore, W., Crouch, E. 1988, CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins. *Biochemistry* 27, 8576-8584

Postle, A.D., Gonzales, L.W., Bernhard, W., Clark, G.T., Godinez, M.H., Godinez, R.I., Ballard, P.L. 2006 Lipidomics of cellular and secreted phospholipids from differentiated human fetal type II alveolar epithelial cells. *J. Lipid. Res.* 47, 1322-31

Postle, A.D., Hunt, A.N. 2009, Dynamic lipidomics with stable isotope labelling. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877, 2716-2721

Postle, A.D., Mander, A., Reid, K.B., Wang, J.Y., Wright, S.M., Moustaki, M., Warner, J.O. 1999, Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. Am. J. Respir. Cell Mol. Biol. 20, 90-98

Postle, A.D., Wilton, D.C., Hunt, A.N., Attard, G.S. 2007, Probing phospholipid dynamics by electrospray ionisation mass spectrometry. Prog. Lipid Res. 46, 200-224

Pynn, C.J., Henderson, N.G., Clark, H., Koster, G., Bernhard, W., Postle, A.D. 2011, Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed *in vivo*. J. Lipid Res. 52, 399-407

Restrepo, C.I., Dong, Q., Savov, J., Mariencheck, W.I., Wright, J.R. 1999, Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. Am. J. Respir. Cell Mol. Biol. 21, 576–585

Schelenz, S., Malhotra, R., Sim, R.B., Holmskov, U., Bancroft, G.J. 1995, Binding of host collectins to the pathogenic yeast *Cryptococcus neoformans*: human surfactant protein D acts as an agglutinin for acapsular yeast cells. Infect. Immun. 63, 3360–3366.

Stymne, S., Stobart, A.K. 1985, Involvement of acyl exchange between acyl-CoA and phosphatidylcholine in the remodelling of phosphatidylcholine in microsomal preparations of rat lung. *Biochim Biophys Acta* 837, 239-250

Todd, D.A., Marsh, M.J., George, A., Henderson, N.G., Barr, H., Sebastian, S., Clark, G.T., Koster, G., Clark, H.W., Postle, A.D. 2010, Surfactant phospholipids, surfactant proteins, and inflammatory markers during acute lung injury in children. *Pediatr. Crit. Care Med.* 11, 82-91

Torday, J.S., Rehan, V.K. 2002, Stretch-stimulated surfactant synthesis is coordinated by the paracrine actions of PTHrP and leptin. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283, L130-L135

Walker, P.R. 1983, The molecular biology of enzyme synthesis. Regulatory mechanisms of enzyme adaptation. In: Walker PR, editor. *The molecular biology of enzyme synthesis. Regulatory mechanisms of enzyme adaptation.* New York: Wiley-Interscience

Wright, S.M., Hockey, P.M., Enhoring, G., Strong, P., Reid, K.B., Holgate, S.T., Djukanovic, R., Postle, A.D. 2000, Altered airway surfactant phospholipid composition and reduced lung function in asthma. *J. Appl. Physiol.* 89, 1283-1292

Zhou, J., You, Y., Ryan, A.J., Mallampalli, R.K. 2004, Upregulation of surfactant synthesis triggers ABCA1-mediated basolateral phospholipid efflux. *J. Lipid Res.* 45, 1758-1767

Figure(s)

Figure 1

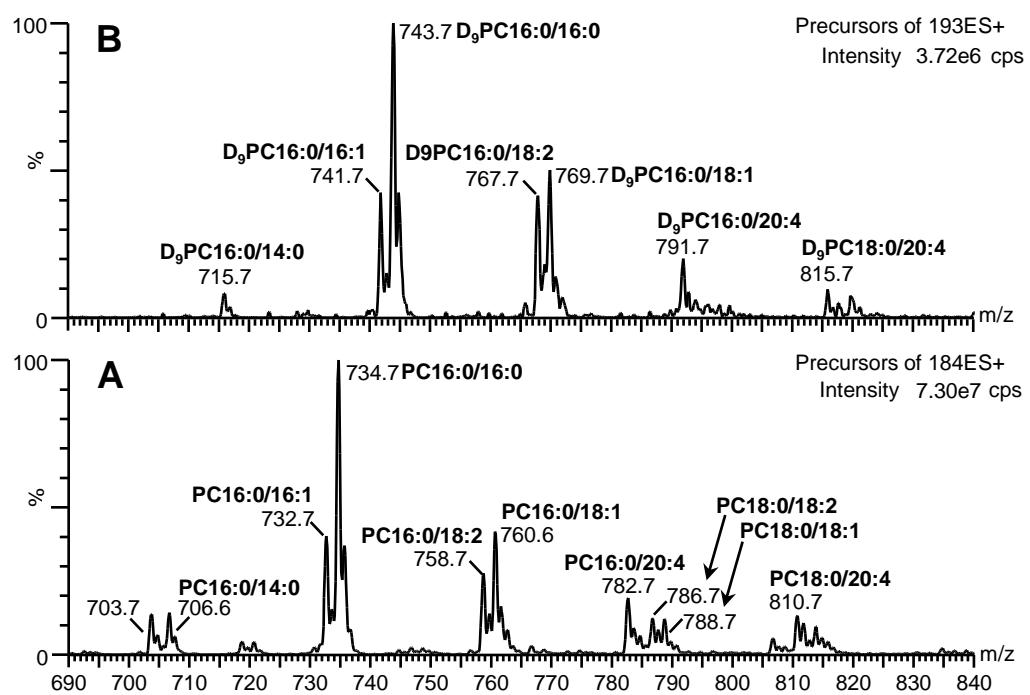


Figure 2

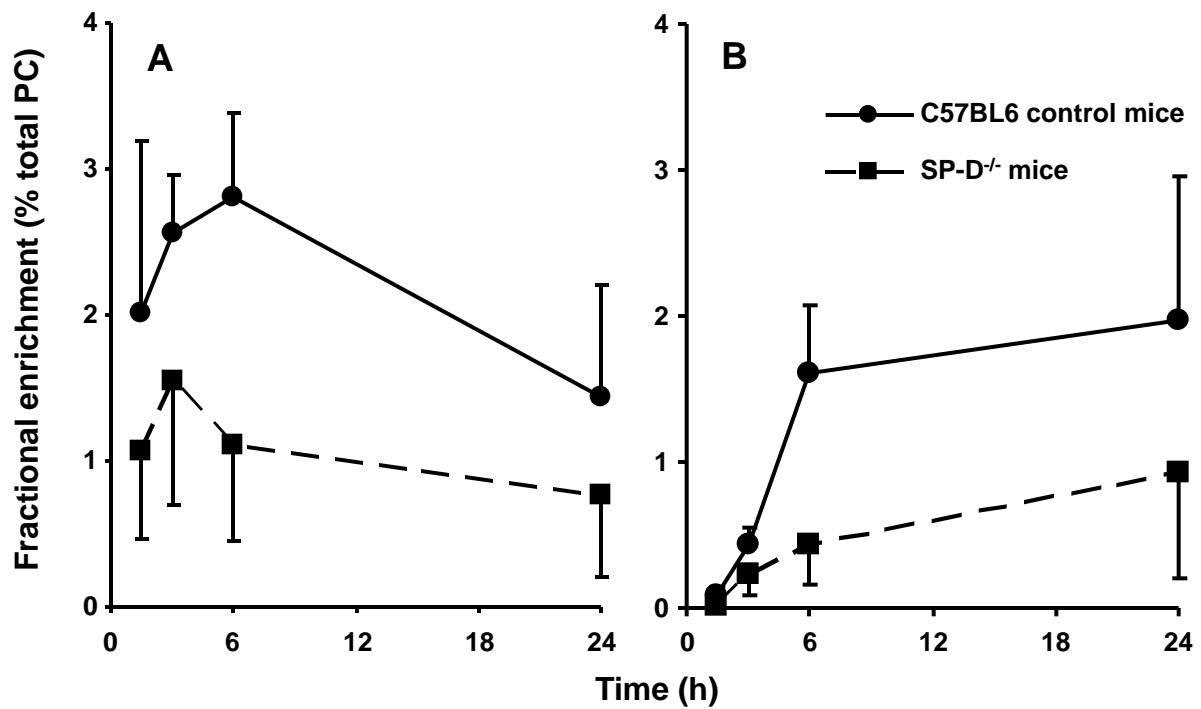


Figure 3

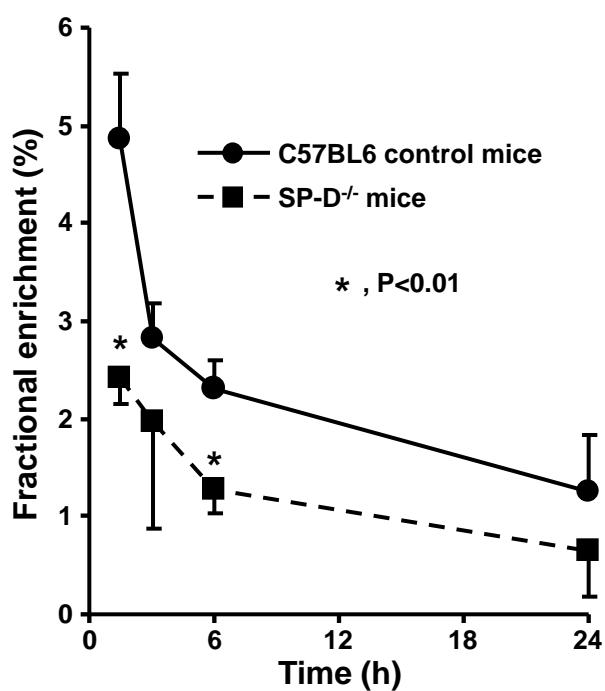


Figure 4

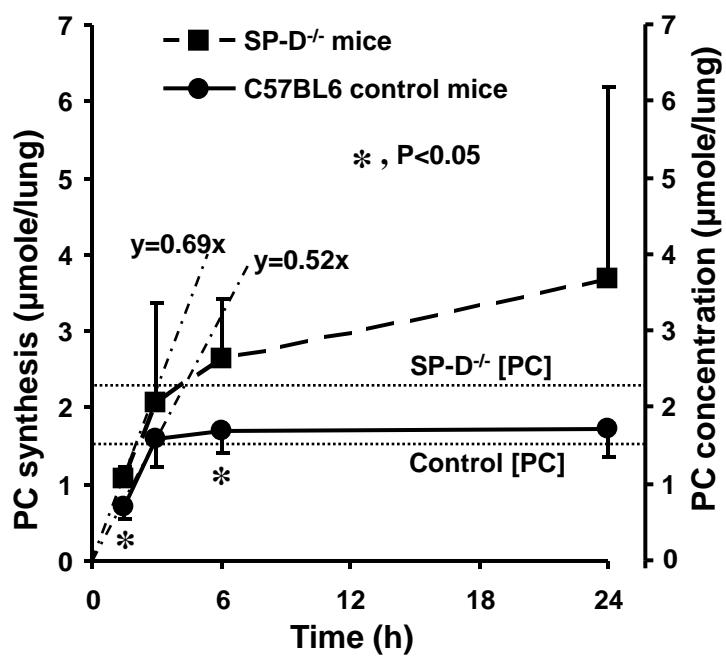


Figure 5

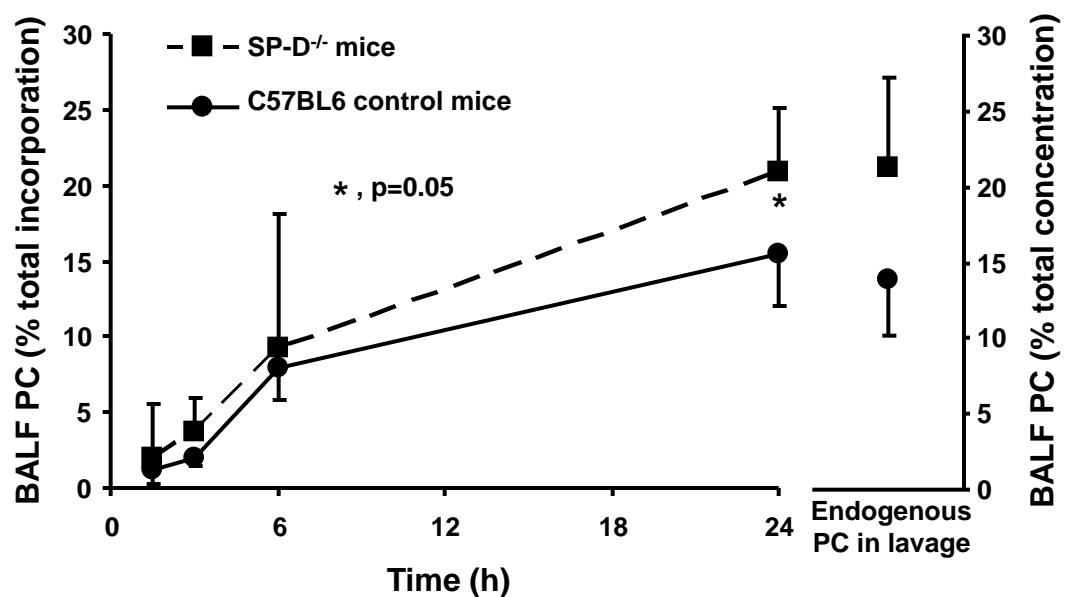


Figure 6

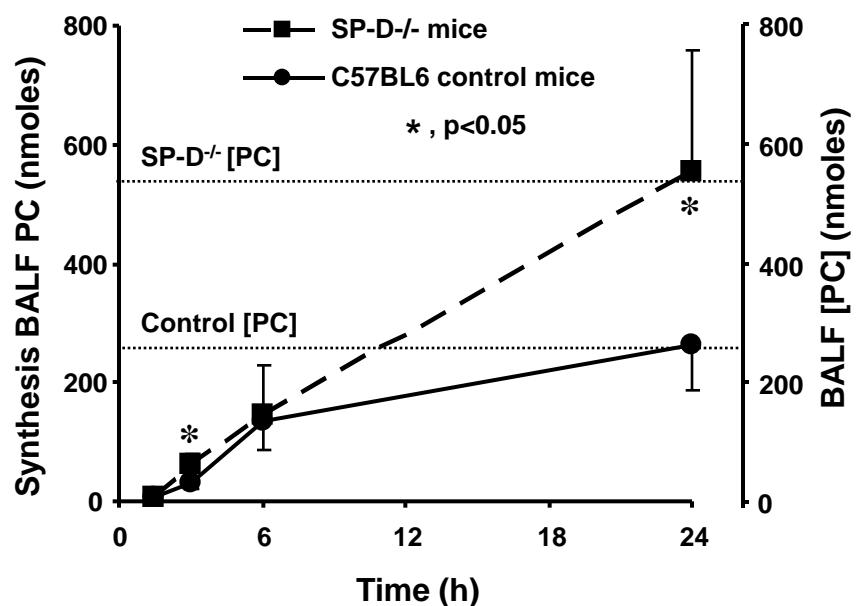
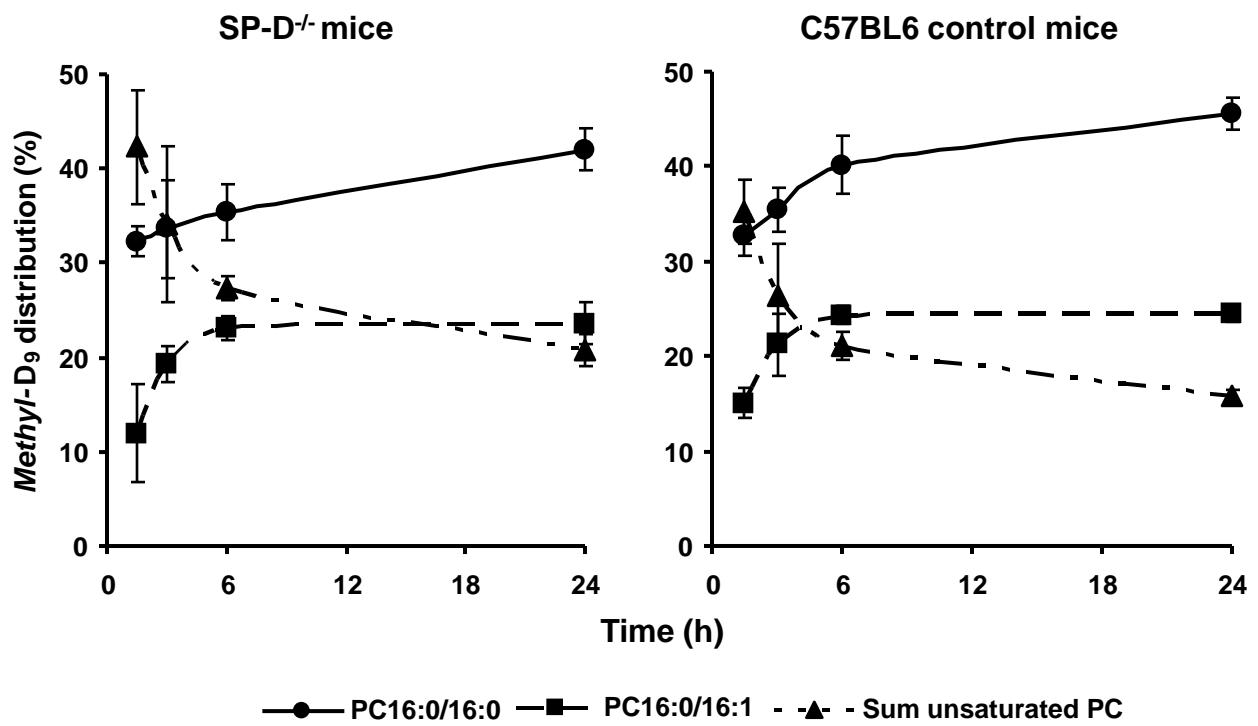
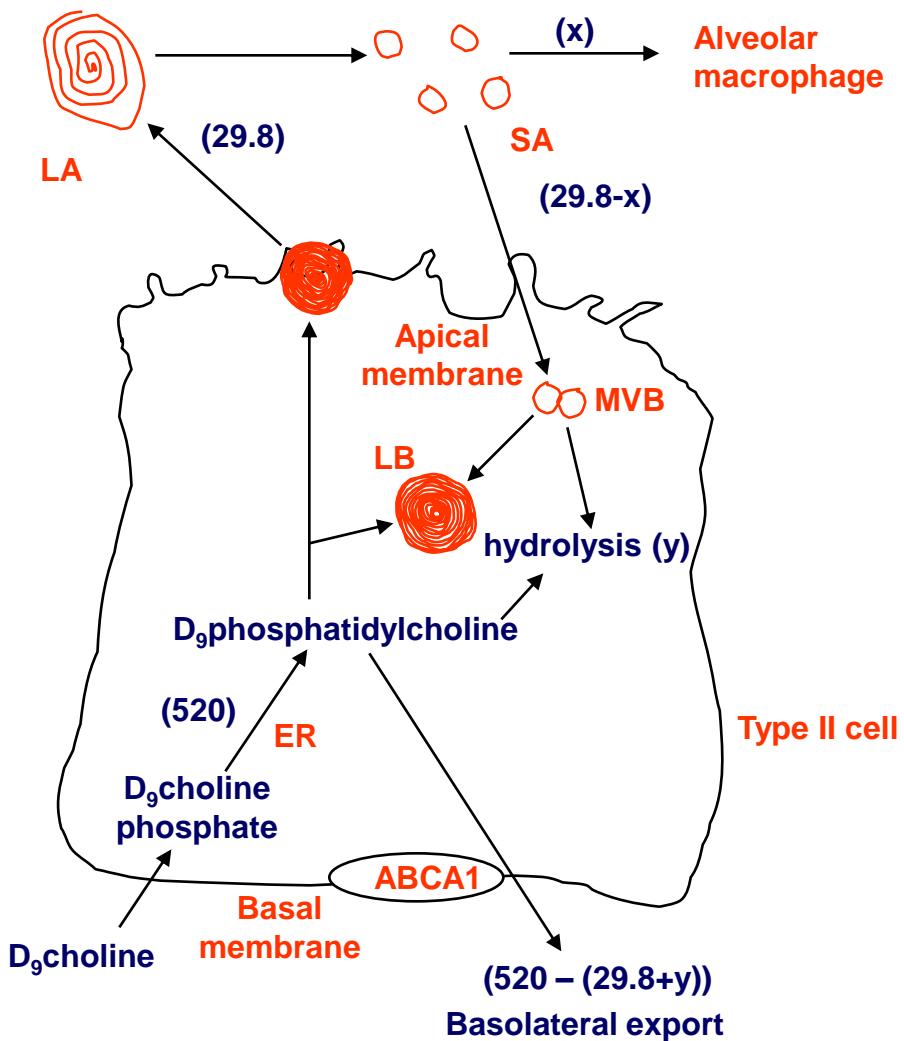


Figure 7



- 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



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 extrahepatic source of circulating, PUFA-enriched PC.