Defective Surfactant Secretion in a Mouse Model of Hermansky-Pudlak Syndrome

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Hermansky-Pudlak syndrome (HPS) in humans represents a family of disorders of lysosome-related organelle biogenesis associated with severe, progressive pulmonary disease. Human case reports and a mouse model of HPS, the pale ear/pearl mouse (ep/pe), exhibit giant lamellar bodies (GLB) in type II alveolar epithelial cells. We examined surfactant proteins and phospholipid from ep/pe mice to elucidate the process of GLB formation. The 2.8-fold enrichment of tissue phospholipids in ep/pe mice resulted from accumulation from birth through adulthood. Tissue surfactant protein (SP)-B and -C were increased in adult ep/pe mice compared with wild-type mice (WT), whereas SP-A and -D were not different. Large aggregate surfactant (LA) from adult ep/pe mice had decreased phospholipid, SP-B, and SP-C, with no differences in SP-A and -D compared with WT. Although LA from ep/pe animals exhibited an increased total protein–to–total phospholipid ratio compared with WT, surface tension was not compromised. Phospholipid secretion from isolated type II cells showed that basal and stimulated secretion from ep/pe cells were $\sim$ 50% of WT cells. Together, our data indicate that GLB formation is not associated with abnormal trafficking or recycling of surfactant material. Instead, impaired secretion is an important component of GLB formation in ep/pe mice.

Keywords: alveolar type II cell; Hermansky-Pudlak syndrome; lamellar body; secretion; surfactant

The lamellar body of alveolar epithelial type II cells is a lysosome-related, intracellular storage organelle for newly synthesized and recycled surfactant components. As such, this organelle interfaces with both the secretory and endosomal pathways within the type II cell. Newly synthesized hydrophobic surfactant proteins, surfactant protein (SP)-B and -C, traffic to (and, in the case of SP-C, complete post-translational processing in) the lamellar body (1), whereas the hydrophilic surfactant protein, SP-A, only enters the lamellar body after it is recycled into the type II cell via receptor-mediated endocytosis (2). Surfactant-specific phospholipids, such as disaturated phosphatidylcholine, are packaged into lamellar bodies through a mechanism that is, at present, poorly understood. The recent description of ABCA3, an ATP-binding cassette transporter, in the limiting membrane of lamellar bodies suggests that this protein may play a role in concentrating phospholipid into lamellar bodies (3).

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Like other lysosome-related organelles, such as melanosomes and platelet dense granules, lamellar bodies undergo a process of maturation and regulated secretion. Maturation is implied from the observation that not all lamellar bodies are released upon secretagogue stimulation (4). Lamellar body secretion is triggered via at least three signaling pathways: activation of adenylate cyclase, protein kinase C, a cAMP-dependent protein kinase, and Ca^{++}-regulated activation of Ca^{++}-calmodulin-dependent protein kinase (reviewed in Ref. 5). Lamellar bodies engage the exocytic machinery in part through the actions of annexin II (6) and α-SNAP/NSF (7), releasing their contents into the alveolar space through pores derived from membrane fusion between lamellar body and plasma membranes (8).

Despite the wealth of information on lamellar body contents and secretion, surprisingly little is known about the process of lamellar body genesis. Electron microscopic studies have suggested that the lamellar body results from redistribution of phospholipid membranes within and repeated fusion with multivesicular bodies (1). These late endosomes develop through the invagination of limiting membrane into the interior of the organelle, and in theory, through the action of the fusogenic SP-B, resulting in lysis of the interior vesicles. The role of SP-B is supported by the presence of multivesicular bodies but not lamellar bodies in mice in which the SP-B gene has been deleted (9), in addition to prior demonstration of the fusogenic potential of SP-B *in vitro* (10). Organelles in transition between multivesicular bodies and lamellar bodies, referred to as composite bodies, have similarly been described within type II cells (1).

Disorders of lamellar body genesis currently fall into two categories: (*1*) failure of production, and (*2*) formation of giant lamellar bodies. In addition to SP-B deficiency (11, 12), impaired lamellar body genesis was observed in patients with neonatal respiratory failure associated with defects in the ATP-binding cassette protein, ABCA3 (13). Giant lamellar bodies have been described in human patients and in animal models. For example, Hermansky-Pudlak syndrome consists of several different genetic defects sharing the common clinical phenotype of oculocutaneous albinism and defective platelet dense granule function. Selected subsets of patients with Hermansky-Pudlak syndrome also exhibit pulmonary pathology, including enlarged foamy type II cells and progressive pulmonary fibrosis, often leading to death (14). The etiology of the severe, progressive pulmonary fibrosis that develops in the fourth decade is unclear (15).

Bred initially for their attractive coat colors, there are several congenic strains of mice notable for varying degrees of oculocutaneous albinism (recently reviewed in Ref. 16). Many of these coat color mutant mice carry genetic defects homologous to mutations in patients with Hermansky-Pudlak Syndrome and phenocopy the human disease with the exception of the pulmonary fibrosis. Instead, the common pulmonary finding in the mouse mutants is progressive emphysema (17). Attempts to mimic the human phenotype using intercrosses enhanced the mouse phenotype but did not result in pulmonary fibrosis. Offspring of intercrosses between

pale ear (involving the HPS1 protein of the BLOC-3 complex) and pearl (involving the Ap3b1 subunit of the AP-3 complex) mice exhibited enlarged foamy alveolar type II cells, inflammation, and reduced survival due to severe emphysema (double mutant ep/ep, pe/pe, herein designated as ep/pe, 18). The observed increases in surfactant components in the tissues of ep/pe mice are consistent with the giant lamellar bodies demonstrated by electron microscopy and with the purported role of these protein complexes in post-Golgi vesicular trafficking.

In this report, we examined the surfactant protein and phospholipid composition of tissues and bronchoalveolar lavage from ep/pe animals to begin to understand the mechanism for giant lamellar body formation in alveolar type II cells. The lamellar body lies at the intersection of the synthetic, lysosomal, and endocytic pathways; therefore, we chose to focus on surfactant components that participate in these pathways to investigate the mechanism of giant lamellar body formation. *In vitro* studies of isolated alveolar type II cells indicate that lamellar body secretion is impaired but not abrogated, thus leading to tissue accumulation and alveolar deficiency of surfactant phospholipid and hydrophobic proteins. Furthermore, the developmental accumulation of phospholipids indicates that this is a lifelong process in ep/pe mice. After demonstrating that synthesis and recycling of surfactant components are essentially intact, we suggest that the impaired secretion of giant lamellar bodies is a problem of lamellar body maturation.

MATERIALS AND METHODS

Animals

Wild type C57BL/6J mice and mutant *Hps1^{ep}/Hps1ep, Ap3b1^{pe}/Ap3b1^{pe}* (ep/pe) mice on the same C57BL/6J background at 10–15 wk old (unless otherwise specified) were all maintained and bred at the Roswell Park Cancer Institute as previously described (19). Male animals were used exclusively for these studies; however, prior work indicated no differences between male and female animals (18). Animals were shipped for surfactant studies as described below and killed within 2 d of transport. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees and adhered to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents

Surfactant antisera used in these studies included rabbit polyclonal antisera to murine SP-A (20), rabbit polyclonal antiserum to mature bovine SP-B (21), rabbit polyclonal antiserum to mature recombinant human SP-C in which Phe was substituted for Cys residues 3 and 4 (Byk Gulden, Altana, Konstanz, Germany), rabbit polyclonal antiserum to rat SP-D (22), and monoclonal antibody to GAPDH (Chemicon, St. Louis, MO). All surfactant antisera reacted against mouse surfactant proteins. A mouse monoclonal antibody directed against rat ABCA3, mAb 3C9, was used for the detection of ABCA3 and was the kind gift of Dr. Henry Schuman. Species-specific, HRP- and Alexa 488–conjugated secondary antisera for immunoblotting were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Preparation and Analysis of Surfactant Fractions from Bronchoalveolar Lavage

WT and ep/pe mice were killed with a lethal dose of pentobarbital and exsanguinated by aortic transection. The trachea was exposed through a midline incision and cannulated with a polyethylene catheter. All lungs were lavaged with 0.5- to 1-ml aliquots of sterile Ca^{2+} and Mg^{2+}-free 0.9% saline to a total of 5 ml instilled and recovered. The bronchoalveolar lavage (BAL) samples were centrifuged using a swinging-bucket rotor at 400 × *g* for 10 min at 4°C, and the cell-free supernatant was separated into large aggregate (pellet; surface active) and small aggregate (supernatant; inactive surfactant and soluble proteins) fractions by a second centrifugation using a fixed angle rotor at 20,000 × *g* for 60 min at 4°C.

Large aggregate (LA) pellets resuspended in saline, and small aggregate (SA) fractions, were reserved for biochemical characterization. The total protein content of the samples from LA and SA fractions was determined with the method of Bradford, with bovine IgG as a standard (23). The total phospholipid content of the samples from LA and SA fractions was determined with the method of Bartlett (24).

Capillary Surfactometer

The capacity of surfactant to maintain airway patency was measured in a capillary surfactometer, which has been demonstrated to simulate terminal human airways as described in detail elsewhere (25). The LA fraction of the BAL was diluted to a concentration of 1 mg/ml phospholipid. Individual LA surfactant preparations (0.5-μl samples) were introduced into the narrow section of a glass capillary and compressed for 120 s, resulting in cyclic extrusion from the narrow end of the capillary permitting airflow and capillary patency. A new capillary is used for each sample evaluated. Dysfunctional surfactant exhibits decreased capillary patency. A microcomputer calculates the percentage of the 120-s study period that the capillary is open to a free airflow. Data used here are the mean of triplicate assays of each LA sample.

Real-Time RT-PCR

RNA was prepared using RNA STAT (Tel-Test, Inc., Friendswood, TX) per the manufacturer's instructions. Samples were then treated with RQ1 RNase-free DNase (Promega, Madison, WI) and ethanol precipitated after phenol-chloroform extraction. Integrity, purity, and concentration of RNA were confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) in the Nucleic Acid Core Facility of the Children's Hospital of Philadelphia. cDNA was synthesized from RNA samples using the SuperScript First-Strand RT-PCR kit (Invitrogen, Carlsbad, CA) using manufacturer's instructions. Real-time PCR reactions using a singleplex format were performed using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) as previously described (26). The following Assay-on-Demand Gene Expression probe sets were used for these studies: SP-A Mm00499170_m1, SP-B Mm00455681_m1, SP-C Mm00488144_m1, SP-D Mm00486060_m1, GAPDH Mm99999915_g1. For each probe set, samples were determined to be in linear amplification range using cDNA standards representing 1–100 ng starting RNA derived from a set of pooled adult C57BL/6J mice to allow for comparisons both within and between experiments.

Western Immunoblotting

Lung samples were homogenized in 1× protease inhibitors (Complete Mini protease inhibitor cocktail; Roche Applied Science, Indianapolis, IN) and centrifuged at 2,000 × *g* to remove debris. Western immunoblotting for SP-A, SP-B, SP-D, and GAPDH was accomplished using previously described procedures (27), using NuPAGE Bis-Tris gels with MES Running Buffer (Invitrogen, Carlsbad, CA) and transferring as per the manufacturer's protocol to Duralose membranes (Stratagene, La Jolla, CA). SDS-polyacrylamide gel electrophoresis for SP-C was performed in 16.5% polyacrylamide gels using a Tris-Tricine buffer system as previously described (28). Total protein loaded per lane was 50 μg for tissue homogenates and 10 μg for LA samples. Specific proteins were visualized by enhanced chemiluminescence using the ECL Kit (Amersham, Inc., Arlington Heights, IL) or Pierce SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and band intensity of exposed film was analyzed by densitometric scanning and quantitated using Kodak 440 image analysis software (Eastman Kodak, Rochester, NY).

Type II Cell Isolation and Phospholipid Secretion Studies

Type II pneumocytes were isolated from five C57BL/6J wild-type or five double mutant ep/pe mouse lungs using dispase, mesh filtration, and panning over IgG-coated and uncoated plates, as described previously (29). The final cell isolates were placed on 35-mm tissue culture dishes (Costar, Cambridge, MA) coated with Type I collagen at 3 × 10^6 cells/dish in Ham's F12 culture medium supplemented with 15 mM HEPES, 0.8 mM $CaCl_2$, 0.25% bovine serum albumin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selinite, and 2% mouse serum. The cells isolated in this manner are > 95% pure type II cells (29). A quantity of 0.5 μCi/dish of (methyl-^{3}H)-choline (Amersham) was added

to the medium to label cellular phospholipids. After 24 h the cells were washed thoroughly and incubated for 30 min in modified Eagle's medium. One set of cells and media were harvested at this time point and served as a control for PC secretion associated with the change of medium, as described previously (30). The remaining cells were incubated without or with the secretagogue, adenosine triphosphate (ATP, 1 mM; Sigma, St. Louis, MO). After 2 h of ATP stimulation, the media were removed and centrifuged to remove detached cells, methanol was added to the cell monolayer, and the cells were scraped from the dish. Both media and cell pellets were extracted for phosphatidylcholine (31). Phospholipid secretion was calculated as the percentage of lipid degradations per minute (dpm) in the medium divided by the total dpm in the cells plus medium. All experiments were performed in duplicate, and the values were averaged.

Immunostaining and Fluorescence Microscopy

Type II cells were fixed and permeabilized with methanol-acetone (1:1 by volume) for 2 min at 4°C, washed with phosphate-buffered saline containing 1 mg/ml bovine serum albumin (PBS-BSA), and incubated with mAb 3C9 (1:250 dilution, 2 h, 4°C). After washing with PBS-BSA buffer, the cells were incubated with Alexa 488–conjugated goat anti-mouse IgG (1:200 dilution; Molecular Probes, Eugene, OR) at 4°C for 1 h. After 5 washes with PBS-BSA buffer the cells were fixed with 4% paraformaldehyde in PBS and examined with an inverted Nikon fluorescent microscope (Nikon, Melville, NY).

Statistical Analyses

Statistical comparisons were made using the unpaired *t* test and two-way ANOVA (Prism 4 Mac version 4.0a; Graph Pad Software, Inc., San Diego, CA), and results are reported as mean ± SE. Results were considered significant at $P < 0.05$ and are reported as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, or NS for $P > 0.05$.

RESULTS

Increased Surfactant Components in Lung Tissue from ep/pe Mice

Total phospholipids in the lung tissue of ep/pe animals were elevated 2.8-fold compared with wild-type 10- to 15-wk-old adult animals (WT 134.7 ± 10.4 versus ep/pe 379.6 ± 39.5 mcg, $***P < 0.001$), as previously noted (18). Examination of animals from birth through 15 wk revealed that tissue phospholipid content was statistically similar between WT and mutant mice on Postnatal Days 0 and 1, followed by a significant, progressive increase in tissue phospholipid that continued into adulthood in the ep/pe mice (Figure 1). Tissue phospholipid levels were nearly doubled by 3 d postnatally in ep/pe mice compared with WT controls.

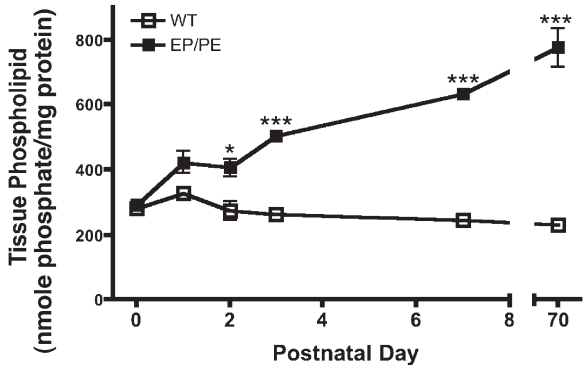

Figure 1. Ep/pe mice exhibit a developmental increase in tissue phospholipid. Total lung phospholipid content was measured from WT and ep/pe animals from Postnatal Day 0 to adulthood (adult = 10–15 wk). Differences in tissue phospholipid between WT and ep/pe mice were evident at Postnatal Day 2 and increased further into adulthood ($*P < 0.05$, $***P < 0.001$, n = 8–20 at each time point).

Both mature hydrophobic surfactant proteins, SP-B and SP-C, were markedly increased in the lung tissue of ep/pe mice (Figure 2), whereas the hydrophilic surfactant proteins SP-A and SP-D were not different from WT animals. The levels of mature SP-C in WT mouse and human lung tissue homogenates are too low to be reliably detected by the SP-C antibody, whereas the antibody consistently detects SP-C in LA surfactant fractions. In keeping with this, the WT lung tissue homogenates exhibited poor immunoreactivity for SP-C. Conversely, the SP-C signal from the ep/pe lung tissue was robust, reflecting a large increase in tissue SP-C in the mutant animals (Figure 2b). The 2.2-fold induction of SP-B in ep/pe lung tissue was statistically significant, but because the SP-C levels in the WT lung tissue were not quantifiable, fold-induction could not be assessed for mature SP-C from ep/pe animals. Despite the increased lamellar body size described previously in ep/pe mice and the observed increases in tissue phospholipid and hydrophobic surfactant proteins, there were no significant differences in RNA for SP-A, -B, or -D by real-time RT-PCR (Figure 3). However, SP-C mRNA was decreased by 32% in ep/pe animals (WT 100.0 ± 3.6% versus ep/pe 67.9 ± 7.7%, $*P < 0.05$).

Altered Surfactant Phospholipid Content of BAL in ep/pe Animals

Enlarged lamellar bodies, a feature of the ep/pe mice, have also been described in the beige mouse model of Chediak-Higashi syndrome, in which the gene defect is in the CHS1/LYST1 gene regulating lysosomal size (32). Because previous studies of beige mice indicated that the lavage phospholipid was decreased concomitantly with increased lung tissue phospholipid (33, 34), we

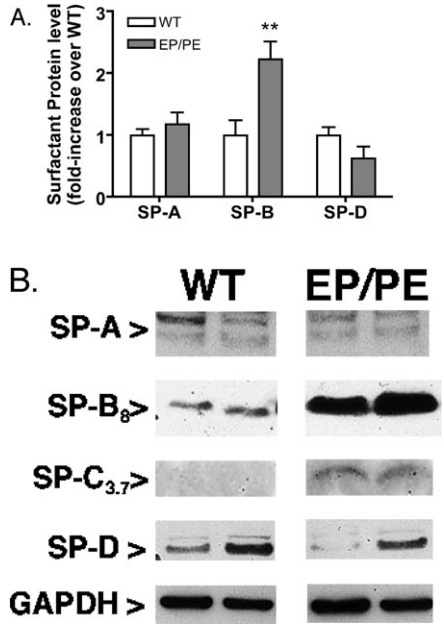

Figure 2. Ep/pe mice exhibit increased tissue hydrophobic surfactant proteins. (*A*) Densitometry results of immunoblots from ep/pe and WT mouse lung homogenates. Data are represented as fold-difference compared with WT after correcting for loading (by GAPDH expression). The ep/pe mouse lung tissue exhibited a 2.2-fold increase in mature SP-B ($**P < 0.01$, n = 5–10). Quantitative analysis of SP-C was not possible due to the poor signal in WT lung homogenates. (*B*) Representative immunoblot lanes from ep/pe and WT mouse lung homogenates for SP-A, -B, -C, and -D, in addition to GAPDH, indicating the dramatic induction of both SP-B and SP-C.

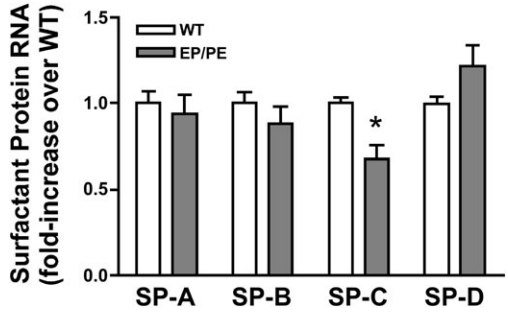

Figure 3. Increased tissue hydrophobic surfactant proteins in ep/pe mice was not due to altered expression of surfactant protein mRNA. Fluorescence signals from real-time RT-PCR of ep/pe and WT mouse RNA were corrected for GAPDH expression to control for loading and then represented as fold-differences compared with WT. Ep/pe mouse lung SP-C RNA was decreased by 32% (*$P < 0.05$, $n = 9$) compared with WT mice. There were no other significant changes in surfactant protein RNA levels between WT and ep/pe mice.

examined total phospholipids from the bronchoalveolar lavage, and large and small aggregate surfactant fractions of individual animals, both WT and ep/pe. We found a 42% reduction in BAL total phospholipid in ep/pe mice compared with WT mice (Figure 4a), in spite of the 2.8-fold increase in tissue phospholipid. The total phospholipid in the large aggregate and small aggregate fractions from the ep/pe animals were proportionately decreased (LA: 44% reduction from WT; SA: 38% reduction from WT).

To examine the quality of the recovered surfactant, we examined both the SA:LA phospholipid ratio and the total protein: phospholipid ratio. The ratio of phospholipid in SA versus LA fractions is an indicator of surfactant function (35), with the ratio increasing as inactivated surfactant accumulates in the SA fraction. We found no difference in the SA:LA ratio (WT 0.58 ± 0.04, ep/pe 0.66 ± 0.11, $n = 5$, NS), suggesting that there was no increased conversion to inactive surfactant in the ep/pe mice. Furthermore, BAL and LA total protein levels were not significantly different in ep/pe and WT animals (Figure 4b), whereas SA surfactant total protein was increased by 2.2-fold compared with WT animals. However, when we examined these results together, ep/pe animals exhibited an increased total protein to total phospholipid ratio in BAL, LA, and SA surfactant fractions (Figure 4c).

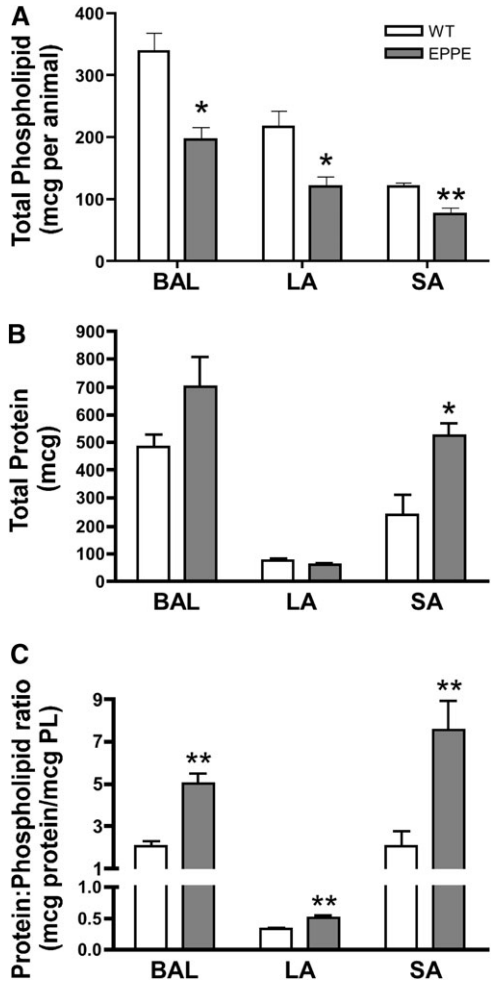

Figure 4. BAL phospholipid levels from ep/pe mice. BAL was fractionated into LA and SA fractions for the determination of total phospholipid per animal. (*A*) Total phospholipid was decreased in the unfractionated BAL, LA, and SA samples from ep/pe mice compared with WT animals. (*B*) Total protein in unfractionated BAL and the LA fraction were not significantly different in ep/pe or WT animals, whereas the total protein was elevated 2.2-fold in the SA fraction. (*C*) The ratio of total protein to total phospholipid was significantly increased in ep/pe mice compared with controls, to 2.5-, 1.6-, and 3.7-fold above WT in BAL, LA, and SA, respectively (*$P < 0.05$, **$P < 0.01$, $n = 5$).

Decreased LA Surfactant Proteins in ep/pe Mice

To complete the characterization of the LA surfactant, we determined the relative abundance in BAL of all four surfactant proteins (Figure 5). When normalized for LA protein, LA SP-B and SP-C were decreased in ep/pe mice to 20.7 ± 5.8% and 37.2 ± 9.0% of WT levels. However, when normalized for LA phospholipid content, the LA SP-B and -C contents in ep/pe mouse LA surfactant were 41 ± 12% SP-B (**$P < 0.01$) and 76 ± 18% SP-C (NS) of WT. By comparison, LA SP-A and SP-D levels (not shown) were not significantly different in ep/pe and WT mice. Some SP-A and the majority of alveolar SP-D are also found in the SA fraction, but there were no differences in SP-A and SP-D levels when we examined SA surfactant fractions from ep/pe and WT mice (not shown). Together, these data suggest that secretion of lamellar body contents is disturbed, resulting in similar reductions in LA phospholipid and SP-C, with a somewhat greater decrease in SP-B, but sparing of the route of secretion for the hydrophilic surfactant proteins.

To determine whether ep/pe mice exhibited altered surface tension properties as a result of these BAL abnormalities, we performed capillary surfactometry on individual LA surfactant samples from ep/pe and WT mice. All samples were corrected to a final concentration of 1 μg/μl phospholipid for these studies. The duration of capillary patency was not significantly different in comparing the ep/pe LA samples to LA surfactant from WT mice (ep/pe 77.4 ± 5.5% open, WT 87.3 ± 3.5% open, $P = 0.15$), indicating no surfactant dysfunction in ep/pe mice.

Decreased Stimulated Secretion by Type II Cells Isolated from ep/pe Mice

The tissue and LA studies of phospholipid and hydrophobic surfactant protein levels suggested a defect of lamellar body secretion in ep/pe type II cells. We isolated alveolar type II pneumocytes from ep/pe and WT animals and examined cellular morphologic characteristics and secretory capacity. To identify

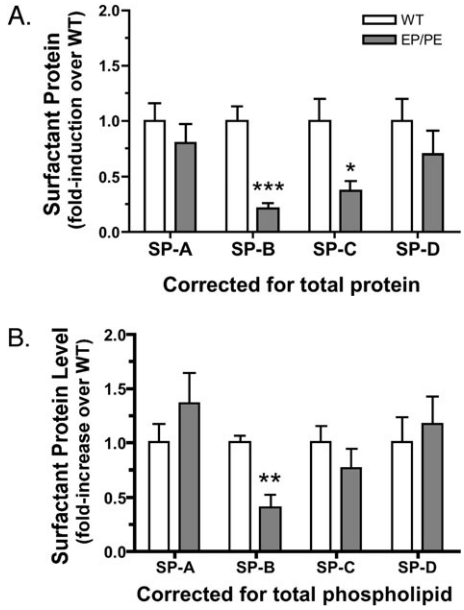

Figure 5. Surfactant protein content of LA surfactant from ep/pe mice. Densitometry results of immunoblots from ep/pe and WT mouse LA surfactant. Data are represented as fold-difference in samples from ep/pe mice compared with WT. (*A*) Surfactant protein content expressed as densitometry units normalized for total protein loaded. (*B*) Surfactant protein content expressed as densitometry units normalized for total phospholipid (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 4$–5).

lamellar bodies we used mAb 3C9, an antibody directed against ABCA3, an ATP-binding cassette transmembrane protein that localizes to the limiting membrane of the lamellar body of type II cells and traffics between lamellar body and plasma membrane (3). As shown in Figure 6, ABCA3 is present in type II cells from both groups of mice. In type II cells from ep/pe mice (Figure 6, *bottom*), ABCA3 is localized to the membranes of both small intracellular vesicles, as is characteristic for lamellar bodies of type II cells from WT mice (Figure 6, *top*), as well as very large vesicles. The labeling of the latter by anti-ABCA3 antibody confirms their identity as enlarged lamellar bodies.

In vitro secretion under basal conditions and in response to secretagogue (1 mM ATP) was examined. Because phosphatidylcholine secretion from C57Bl/6 mice type II cells is linear over a 1- to 4-h time period (36), secretion was examined over a 2-h stimulation period. Incorporation of labeled choline into phosphatidylcholine was not different in type II cells from ep/pe or WT mice (428 ± 43 versus 414 ± 86 cpm/mcg protein, respectively, $P = 0.875$). Unstimulated release (basal) from ep/pe type II cells was 38% of WT type II cells, whereas stimulated release (ATP) from ep/pe type II cells was 46% of WT cells (Figure 7A). WT cells exhibited a significantly increased PC release upon ATP stimulation (1.73-fold). As also shown in the representative time course (Figure 7B), although ep/pe type II cells were responsive to ATP stimulation, the increase above basal secretion was not statistically significant.

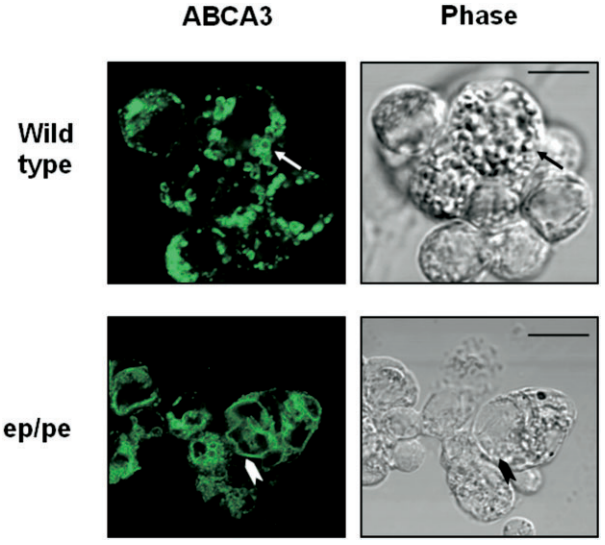

Figure 6. ABCA3 is present in membranes surrounding giant lamellar bodies in ep/pe mice. Representative immunofluorescence (*right*) and phase contrast (*left*) micrographs of isolated type II cells from the lungs of WT (*top*) and ep/pe (*bottom*) mice. ABCA3 is in the limiting membranes of small (*small arrow*) and giant (*large arrow*) lamellar bodies that are also evident by phase contrast microscopy (*bar* = 10 μm).

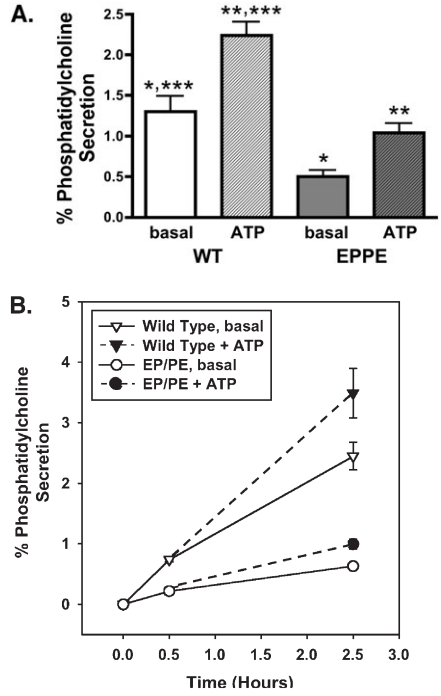

Figure 7. Phospholipid secretion from isolated ep/pe and WT type II cells. Type II cells were incubated overnight with ^{3}H-choline, washed, and incubated without additions (basal) or with 1 mM ATP for 2 h. Percent phospholipid secretion was calculated based upon recovery of ^{3}H-labeled phosphatidylcholine (PC) from culture media and expressed as a % of the sum of the total labeled phospholipid recovered in the type II cells plus the media (means ± SE of $n = 3$–6 independent experiments performed in duplicate). (*A*) Unstimulated (basal) and stimulated secretion from WT and ep/pe type II cells (means ± SE of $n = 3$–6 independent experiments performed in duplicate). Although ep/pe type II cells were responsive to ATP stimulation, the effect was not significant by two-way ANOVA with Bonferonni post-test analysis (2.01-fold, NS, $n = 3$). *Significantly different from WT, basal, $P < 0.05$; **Significantly different from wild type, ATP-stimulated, $P < 0.01$; ***Significantly different from basal, $P < 0.001$. (*B*) Representative time course of secretion from WT and ep/pe type II cells. ($n = 3$–6 determinations from a single experiment; *SE bars* where not visible are within symbols). WT, wild type; EPPE, ep/pe mice.

DISCUSSION

We undertook these studies to characterize surfactant metabolism in the ep/pe mouse as a model of Hermansky-Pudlak syndrome, and we found that surfactant components were indeed altered in the ep/pe mouse lung. Giant lamellar body formation was associated with increased tissue phospholipids, as previously shown (18), and increased tissue content of the mature hydrophobic surfactant proteins SP-B and SP-C. Conversely, alveolar content of phospholipids and mature SP-B and -C proteins were decreased in ep/pe mice compared with WT animals. Furthermore, there were no abnormalities of SP-A or -D in either lung tissue or alveolar surfactant in the ep/pe animals. *In vitro* studies showed that giant lamellar body formation was also associated with decreased basal and stimulated secretion of surfactant. Together, these data strongly suggest that impaired lamellar body secretion is an important component of giant lamellar body formation in ep/pe mice.

There are several possible mechanisms for giant lamellar body formation. Lamellar bodies are lysosome-like storage organelles derived from multivesicular bodies, receiving protein, phospholipids, and other constituents via the secretory, lysosomal, and endocytic pathways. Therefore, abnormal trafficking of lamellar body contents to the lamellar body, excessive reuptake into the type II cell, defective intracellular transport to the plasma membrane, defective secretion at the plasma membrane, and abnormal lamellar body maturation might all result in giant lamellar body formation.

Lamellar body formation is poorly understood, but several aspects of this process have been well described. SP-B deficiency, either due to a primary genetic defect or due to protease inhibition, impairs lamellar body formation from its precursor, the multivesicular body. In association with this defect, SP-C is incompletely processed leading to intracellular and extracellular accumulation of 7- to 10-kD intermediates of SP-C. Despite defective lamellar body formation, tissue and alveolar phospholipid content are minimally disturbed (37). The gene defect in the ep/pe mice compromised neither post-translational processing of SP-B and -C nor trafficking of the hydrophobic surfactant proteins to lamellar bodies, as determined by analysis of the lung tissue. In addition to verifying anterograde transport of SP-B and -C, these data also imply the proper trafficking of lysosomal enzymes required for the proteolytic processing of the hydrophobic surfactant proteins. In the ep/pe mice, the mature SP-B and SP-C accumulate because they are not being secreted. The previous report of this mouse model indicated that proSP-C was also increased (18). Because some of the processing events in SP-C biosynthesis occur within the lamellar body, it is reasonable that both proSP-C and mature SP-C proteins might be increased in the type II cells of ep/pe mice. Interestingly, although we found no impairment of SP-B mRNA, we noted a 32% decrease in SP-C mRNA associated with the accumulation of these hydrophobic proteins in ep/pe mice, suggesting that there may be an attempt to downregulate further production of SP-C. Given the recently described toxicity of aberrant SP-C proteins (38), it is possible that the accumulation of SP-C plays a role in the pulmonary pathophysiology in the ep/pe animals.

We also found that the important lamellar body membrane protein ABCA3 was located in the membranes of the lamellar bodies in type II cells isolated from ep/pe mice. ABCA3, an ATP-binding cassette protein, localizes to the limiting membrane of the lamellar body and traffics between lamellar body and plasma membrane (3). Recently, ABCA3-deficient patients with neonatal respiratory failure were shown to have small lamellar bodies, data supporting a possible role of ABCA3 in lamellar body formation (13). The presence of ABCA3 in the limiting membrane of lamellar bodies of ep/pe mice indicates that anterograde trafficking of this lamellar body constituent is also normal in ep/pe mice. Finally, our *in vitro* studies indicate that phospholipid synthesis is similar in both WT and ep/pe mice, indicating that the formation of giant lamellar bodies is not due to overproduction of phospholipids destined for lamellar body storage. Together our data indicate that it is unlikely that giant lamellar body formation is due to abnormal anterograde trafficking of lamellar body constituents.

Up to 80% of alveolar surfactant is recycled back into type II cells for repackaging into lamellar bodies. Therefore, enhanced reuptake of surfactant components could explain both the tissue accumulation and alveolar deficit in the ep/pe mice. One of the primary modes of recycling surfactant into the type II cell involves the internalization of SP-A and lipid via a coated pit pathway into an early endosome compartment, possibly through interaction with an SP-A receptor (39, 40). SP-A recycles back to the surface of the cell and is resecreted while lipid is transferred to lamellar bodies (40, 41). If inhibition of this pathway were a primary mechanism for giant lamellar body formation, then SP-A would be expected to accumulate in the lung tissue and become depleted from the BAL of ep/pe mice. Instead we found that both hydrophilic surfactant proteins SP-A and SP-D were unaffected in lung tissue and BAL from ep/pe mice.

Lamellar body maturation is also poorly understood; however, it is well known that upon a secretory stimulus, only a fraction of lamellar bodies in type II cells are secreted. Surfactant release after sustained exposure of type II cells to secretagogues is linear over 4 h and then plateaus, indicating that the lamellar bodies left within the type II cell are not yet capable of secretion (36). We have shown that both basal and stimulated secretion were decreased in the isolated type II cells of ep/pe mice. The 50% reduction in basal and stimulated phospholipid secretion from type II cells is consistent with our observed decrease in total LA surfactant phospholipid, SP-C and SP-B, all of which are secreted in concert from lamellar bodies (42). Importantly, secretion of lamellar bodies is not abolished in the ep/pe mice. The presence of surfactant in the BAL with normal phospholipid:hydrophobic protein ratios, despite reduced alveolar content, is evidence for lamellar body secretion. Although alternate pathways such as decreased catabolism or enhanced uptake of phospholipid components have not been ruled out, our studies strongly indicate that impaired maturation of lamellar bodies resulting in reduced secretion in the ep/pe mice is a major contributing factor in the tissue accumulation and BAL deficit of surfactant.

The defect in the ep/pe pulmonary system is reminiscent of the reduced secretion of lysosomes in the beige mouse model of Chediak-Higashi syndrome, involving mutation of the CHS1/LYST1 gene (32). Beige mice develop foamy alveolar type II cells due to the presence of numerous large lamellar bodies, on average approaching 4-fold increased lamellar body diameter compared with control animals (43). Alveolar lavage DSPC pool size in beige mice was reduced to 60% of controls (34), similar to the 50% reduction in phospholipids we observed in the ep/pe mice. Although lamellar body secretion from type II cells was not directly studied in the beige mouse, defective secretion of enlarged fibroblast (44) and kidney (45) lysosomes has been demonstrated, with remarkable similarity to the decreased lamellar body secretion we observed from the ep/pe mouse type II cells. CHS-1/LYST1 regulates lysosome size, and overexpression in fibroblasts is associated with small lysosomes, whereas the knockout phenotype results in giant lysosomes (reviewed in Ref. 46). In studies of macrophages isolated from beige mice, smaller lysosomes from beige mouse macrophages were capable of transport along microtubules while the giant lysosomes were not (44). Importantly, this defect could

be duplicated in normal macrophages after phagocytosis of enlarged beads, suggesting that the lysosome size is a critical determinant in lysosomal transport. Previous studies of the secretion of kidney lysosomes indicated defective secretion in ep/ep and pe/pe mice (47). Although similar studies have not been undertaken in the ep/pe mice, the variability in lamellar body size and our observation that lamellar body secretion was impaired but not abolished in ep/pe mice are consistent a defect in transit to the plasma membrane of giant lamellar bodies.

Regardless of mechanism, giant lamellar body formation is associated with progressive lung pathology in the ep/pe mice. Our data show that tissue phospholipid accumulation from the resulting giant lamellar bodies begins early in postnatal life and continues into adulthood as has been seen in gene-targeted SP-C and SP-D mice (48–50). The observed pathology is unlikely due to the decreased BAL phospholipids, SP-B and SP-C, as we observed normal surface tension of the LA surfactant fraction. Although SP-B levels were decreased by $> 50\%$ in the ep/pe mouse LA fraction, capillary surfactometry was unchanged. This was not surprising in light of studies by Ikegami and colleagues showing that the presence of adequate SP-C can compensate for a paucity of SP-B in unstressed, transgenic mice (50). Thus, although the levels of surfactant are reduced, there is a sufficient quantity of normal material to maintain adequate respiratory function. Although there is evidence for the presence of lung inflammation and emphysema (18), further studies are needed to completely characterize the pulmonary complications in this mouse model as well as in patients with HPS.

In summary, the results demonstrate that the cellular defect in the ep/pe mice does not interfere with synthesis of lamellar body contents or the initial transfer of these components into the lamellar body. Recycling is likewise normal in the ep/pe mice. Secretion is significantly impaired but not abrogated in this mouse model, indicating that, if able to engage the transport machinery, lamellar bodies are capable of secretion. One remaining cellular process that could explain giant lamellar body formation in the ep/pe mouse is the poorly understood area of lamellar body maturation, particularly events that regulate organelle size. In this way, the ep/pe mice appear to be similar to the CHS1/beige mice, in which dysregulation of the size of lysosomes and lysosome-related organelles impedes access to cellular transport, thus leading to impairment of secretion. Further studies elucidating the processes involved in lamellar body maturation will aid in the understanding of the mechanism of giant lamellar body formation in the ep/pe mouse and in patients with HPS.

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