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Protein–Lipid Interactions and Enzyme Requirements for Light Subtype Generation on Cycling Reconstituted Surfactant[1]

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Surfactant convertase is required for conversion of heavy density (H) natural surfactant to light density (L) subtype during cycling *in vitro*, a technique that reproduces surfactant metabolism. To study mechanisms of H to L conversion, we prepared liposomes of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG), or the phospholipids (PL) in combination with either surfactant protein A (SP-A), surfactant protein B (SP-B), or both SP-A and SP-B. Phospholipids alone showed time-dependent conversion from heavy to light subtype on cycling in the absence of convertase, which was decreased by adding SP-B, but not SP-A, to phospholipids ($p<0.01$ for PL+SP-B, or PL+SP-A+SP-B vs. PL, or PL+SP-A). The ultrastructure, surface activity, buoyant density, and L subtype generation on cycling PL+SP-A+SP-B with partially purified convertase or with phospholipase D were similar to those of natural TM. In conclusion, a reconstituted surfactant mimics the behavior of natural surfactant on cycling, and reveals that interaction of SP-B with phospholipids decreases L subtype generation. In addition, esterase/phospholipase D activity is required for conversion of heavy to light subtype on cycling. © 1998 Academic Press

Key Words: **surfactant; surfactant metabolism; surfactant artificial; surfactant proteins; surfactant convertase.**

The secular forms of pulmonary surfactant within the alveoli include: heavy density subtypes containing predominantly lamellar bodies (LBs) and tubular myelin (TM); and light density subtypes composed of small vesicles (SVs) (1). The heavy subtypes contain surfactant apoproteins and rapidly generate a monolayer which lowers surface tension at the air/fluid interface (1). SVs, derived from phospholipids squeezed out of the surface film during compression, lack surfactant apoproteins and surface active properties (1-4). The metabolic transformation of heavy to the light subtypes can be reproduced *in-vitro* by cyclic expansion and contraction of the surface of a suspension of nascent surfactant (TM), a process called cycling (5). Furthermore, conversion of TM to SV requires an enzyme, surfactant convertase, present in alveolar lavage (6,7). Convertase has recently been shown to be a novel carboxylesterase with a serine active site whose action is inhibited by diisopropylfluorophosphate (DFP) (8). A purified porcine liver carboxylesterase also reproduces conversion of heavy to light subtype on cycling (8), thereby supporting the view that esterase activity is important for light subtype generation.

The complex nature of natural TM makes it difficult to determine the changes occurring during its transformation to SV. Reconstituted surfactants containing synthetic phospholipids and purified surfactant proteins mimic the properties of natural TM (9-11). Barr and co-workers concluded that the surfactant apoproteins were not the source of convertase activity because cycling reconstituted surfactants in the absence of convertase did not generate light subtype (11). After several hundred cycling experiments, using different phospholipid mixtures and varying concentrations of SP-A and SP-B, we prepared a reconstituted surfactant that mimicked the cycling properties of natural TM by converting to light subtype on cycling with a partially purified convertase prepared from mouse alveolar lavage. We found that SP-B, in much lower concentrations than those used by previous workers (9-11), decreased light subtype generation during cycling, and light subtype generation on cycling a reconstituted surfactant containing both SP-A and SP-B required esterase or phospholipase D activity.

MATERIALS AND METHODS

Normal CF1 female mice (Charles River Labs, Wilmington, MA), 15-25 wk old, housed under normal laboratory conditions were used for the experiments. Isotopes were obtained from New England Nuclear (Boston, MA). Reagents and enzymes were obtained from Sigma (St. Louis, MO) or Fisher Chemicals (Itasca, IL). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Polyclonal rabbit antibodies to human SP-A or bovine SP-B were graciously gifted by Dr. Jeffrey Whitsett (University of Cincinnati, Cincinnati, OH).

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We used a mixture of dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylglycerol (PG; phosphatidyl-DL-glycerol, sodium salt), SP-A and SP-B were purified from alveolar lavages of patients with alveolar proteinosis or, in some experiments, from pooled mouse alveolar lavages (12-14). The synthetic phospholipids in combination with purified SP-A and SP-B were cycled with and without convertase. Partially purified convertase for these experiments was obtained from mouse alveolar lavage and nascent secretion by affinity chromatography with concanavalin A sepharose (Con-A). The details of the experiments are as follows:

Isolation of Surfactant from Alveolar Lavage

The first two liters of fluid return, obtained from patients with alveolar proteinosis undergoing whole lung lavage for therapeutic purposes, were collected and phenylmethylsulfonylfluoride (PMSF) was added (final concentration 0.25 mM). Surfactant was isolated using NaBr density centrifugation by the method of Gross & Schultz (7) and stored at −20°C. This purified surfactant was used for isolation of SP-A and SP-B.

Isolation of SP-A

SP-A was purified from the surfactant as previously described (12,13). The protein concentration of the supernatant containing SP-A was measured, and its purity confirmed by SDS-PAGE (15). Silver staining of the gels (Daiichi, Tokyo, Japan) revealed bands corresponding to Mr of ~69 kD and ~35 kD. The identity of SP-A was confirmed by western blotting using an anti-human SP-A antibody.

Isolation of SP-B

SP-B was isolated from the dried surfactant by sequential differential extraction in organic solvents by the method of Beers et al (14). The protein concentration of the purified SP-B was measured, and its amino acid composition confirmed (Worcester Foundation for Biomedical Research, Shrewsbury, MA). SP-B in the extract was separated by 16% tricine gel electrophoresis (16), and presence of ~8 kD monomeric and ~16 kD dimeric forms of SP-B was confirmed by silver staining SDS-PAGE gels, and by western blotting.

Preparation of Reconstituted Surfactant

Phospholipid. DPPC and PG (7:3 w/w) were mixed in chloroform. The phospholipid mixture was incubated with 0.1 μCi [^{14}C]DPPC/mg of the phospholipid mixture at 37°C for 60 min ([dipalmitoyl-1-^{14}C]-PC, 80-120 mCi/mmol, Du Pont NEN).

Addition of SP-B. Radiolabeled phospholipids (10 mg) and SP-B (2-20 μg) were mixed in chloroform, the organic solvents evaporated at 43°C for 10 min and the residue suspended in 3 ml of buffer (2% octylglucopyranoside (OGP) in Tris/NaCl/EDTA) at 57°C. OGP was removed by dialysis against 5 mM Tris, pH 7.4 for 48 h at 4°C. Various concentrations of SP-B (0.02 to 2.0%) were tested, and surfactants containing concentrations of SP-B used in these experiments (0.02 to 0.2% of PL, w/w) mimicked the cycling behavior of natural surfactant most closely.

Addition of SP-A. After dialysis, the phospholipid content of the surfactant was determined by its absorbance at 280 nm (DU 64 Spectrophotometer, Beckman Instruments, Fullerton, CA). Before each cycling experiment, SP-A (50 μg/mg phospholipid), calcium chloride and magnesium chloride were added (final concentration of 1 mM and 2 mM, respectively) and the mixture incubated at 37°C for 1 h.

Preparation of natural heavy surfactant from mouse alveolar lavage. [^{3}H]-Choline labeled natural heavy subtype of surfactant was obtained from mouse alveolar lavage and secretion as previously described (7). This surfactant, used as a substrate in the cycling assay, is mostly composed of aggregates of tubular myelin (6).

Determination of surface activity. The surface activity was measured in a modified all-Teflon Wilhelmy balance with a movable Teflon barrier (Kimray Inc. Oklahoma City, OK). The balance was calibrated on each occasion and all measurements were made at 37°C and 100% humidity. Aliquots containing approximately 250 μg of PL+SP−A+SP-B or natural heavy sub-type surfactant were applied below the surface of 50 ml of cleaned AL buffer (0.15 M NaCl, 5 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid (HEPES), 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4). The sub-phase was stirred continuously at maximal surface area for 10 min. The surface was compressed once from 100 to 15% of the maximum area, while surface tension was recorded. The procedure was repeated, and measurements of surface tension were recorded again during the third compression.

Electron Microscopy

Reconstituted surfactants were processed for electron microscopy according to Williams et al (10). Each surfactant (1-2 ml) was fixed in suspension in 2.5% glutaraldehyde, 1% tannic acid in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h at room temperature. The surfactants were then pelleted by centrifugation at 10,000 × g and stored overnight in fixative at 4°C. The pellets were then washed twice in 0.1M sodium cacodylate buffer, pH 7.4 at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C, dehydrated in acetone, and embedded in 812 resin (Electron Microscopy Sciences). Thin sections (70-80 nm) were stained with uranyl acetate and lead citrate. Sections were examined and photographed with a Hitachi H600 transmission electron microscope.

Preparation of Convertase

Alveolar lavage and nascent secretion obtained from mouse lungs (7) was spun at a low speed to sediment cells (250 × g for 10 min), and the supernatant incubated with Concanavalin A sepharose (Con-A) (Pharmacia LKB, Piscataway NJ; 1:20 v/v) for 40 min at 4°C. The supernatant (void) obtained after centrifugation was discarded, the pellet washed twice with AL buffer, and the bound protein eluted with 25 mM methyl α-D manno-pyranoside in AL buffer (10% of the volume of alveolar lavage). The protein content of the yield was ~50 μg per mouse. The Con-A yield from 5-6 mice was stored in small aliquots at −20 °C and used as the source of convertase. Fresh preparations of the Con-A yield were prepared every 2 weeks for the cycling experiments. To characterize the DFP-binding protein in the Con-A yield, we incubated it with 0.1 μCi [^{3}H]-DFP/ml at 37°C for 30 min ([1,3 ^{3}H]-DFP, >3.0 Ci/mmol, DuPont NEN). Proteins were precipitated with twice the volume of 100 % acetone in the cold for 30 min and sedimented at 2,200 × g for 30 min at 4°C in a Sorvall RT 6000B centrifuge. A portion of the pellet resuspended in AL buffer was electrophoresed through 7.5% polyacrylamide gels according to Laemmli (15). The gels were stained with Coomassie Blue R-250, destained, dried, and exposed to Kodak XAR-2 film (17).

Cycling

We used approximately 100 μg of phospholipid in each tube with the total volume made up to 2 ml with AL buffer, and cycled the tubes for 4 h at 37°C (5). Partially purified convertase (Con-A yield; ~30 μg of protein), alveolar lavage (~50 μg protein), or various phospholipases (5 units/ml) were added to the mixture in appropriate cycling tubes. The purpose of these experiments was to determine the proportion of heavy surfactant that converted to light subtype under cycling conditions. A typical cycling experiment consisted of an uncycled tube to determine the buoyant density of the starting material, tubes cycled without any added enzyme, and tubes cycled with enzyme activity. In some experiments, enzyme activity was inhibited with DFP (10 mM final concentration), by boiling convertase, or with Orlistat (50 μM final concentration). For the experiments with Orlistat, equal amounts of dimethylsulfoxide (DMSO) were added in the

test and the control tubes. Following cycling, the cycling mix was centrifuged to equilibrium in continuous sucrose gradients (0.1 - 0.9 M) at 190,000 × g in a SW 55 rotor at 8°C for 20 h, fractionated, scintillation fluid added to each fraction, and radioactivity determined (7). In a few confirmatory experiments, we determined that the distribution of radioactivity corresponded to the distribution of phospholipid in the various fractions by measuring inorganic phosphorus in the phospholipids extracted from each fraction (18,19).

Protein concentration was measured by the Bradford method (20) using bovine serum albumin as the standard.

Statistical Analysis

Surfactant subtypes were separated by centrifugation on continuous sucrose gradients. Phospholipid peaks generated by cycling were analyzed with computerized software (PeakFit, Jandel Scientific, San Rafael, CA). The buoyant densities and percentage area of each radiolabeled peak was determined, and mean ± SD calculated (5). Differences in light subtype generation following cycling with different enzymes and their inhibitors were determined by the Students t test and the results of various surfactants were compared by ANOVA. Statistical significance was accepted with $p<0.05$.

RESULTS

Determination of Surface Activity

The surface activities of reconstituted surfactant containing PL+SP-A+SP-B and natural heavy subtype of surfactant were compared in 3 paired experiments. Both surfactants lowered surface tension to ~5 mN/m at minimum surface area. The shape of the surface area-surface tension loop was identical in the two surfactant preparations.

Electron Microscopic Studies

The morphology of the various surfactants is shown in Fig. 1. Phospholipid liposomes consisted mainly of bilayered vesicles (≥150 nm) in size. Addition of SP-A produced aggregation of the vesicles into concentric bilayers, whereas addition of SP-B resulted in the formation of sheets of multilamellar membranes. Both SP-A and SP-B in combination with phospholipids formed multilamellar vesicles interspersed with few areas showing formation of lattice-like structures.

Characterization of the Con-A Yield

SDS-PAGE analysis of the Con-A yield revealed a number of protein bands; however, only one DFP-binding protein was identified in the autoradiogram (Fig. 2).

Cycling with Phospholipids Alone (PL)

In the absence of cycling, phospholipids had a peak buoyant density of 1.058 ± 0.002 g/ml (mean ± SD) (Fig. 3). On cycling, there was a time-dependent shift in buoyant density to light subtype. The mean buoyant densities of the peaks were 1.046, 1.038 and 1.030 g/ml after cycling for 1, 2 and 4 h, respectively ($n = 3$) (Fig. 4). The peak buoyant density of phospholipids cycled for 4 h was significantly lower compared to uncycled phospholipids ($p < 0.0001$). In contrast, the buoyant density of phospholipids prepared by sonication (21) varied over a wide range, with the majority being of the light subtype but lacking a distinct light peak. On cycling, sonicated phospholipids did not show a significant shift in buoyant density (data not shown).

Cycling Phospholipids with SP-A (PL+SP-A)

Uncycled PL+SP-A produced a single peak with buoyant density of 1.07 ± 0.003 g/ml (Fig. 3). On cycling, the buoyant density shifted with a peak at 1.036 ± 0.001 g/ml. No definite heavy density peak could be identified after cycling in most experiments. Therefore, SP-A alone did not influence cycling properties of phospholipids.

Cycling Phospholipids with SP-B (PL+SP-B)

The peak buoyant density of uncycled PL+SP-B was 1.063 ± 0.004 g/ml (Fig. 3). On cycling, the phospholipids resolved into two peaks with buoyant densities of 1.062 ± 0.002 and 1.038 ± 0.002 g/ml, respectively. The buoyant densities of the heavy peaks of this surfactant before and after cycling were similar ($p>0.05$). The proportion of light subtype generated on cycling PL+SP-B was significantly lower than that observed on cycling PL or PL+SP-A ($p < 0.001$ for each surfactant). Thus, SP-B decreased light subtype generation compared to that observed on cycling phospholipids alone.

Cycling Phospholipids in Combination with SP-A and SP-B (PL+SP-A+SP-B)

Uncycled PL+SP-A+SP-B produced a single heavy peak (buoyant density 1.074 ± 0.001 g/ml) (Fig. 3). On cycling, the phospholipids resolved into two peaks with buoyant densities of 1.065 ± 0.002 and 1.047 ± 0.003 g/ml, respectively (Fig. 3). Addition of convertase to the cycling mix significantly increased the amount of phospholipid in the light peak compared to cycling in the absence of convertase ($p=0.02$) (Table 1). Similar results were obtained by cycling PL+SP-A+SP-B with alveolar lavage (50 μg of protein), or when surfactant was reconstituted with SP-A and SP-B derived from mouse alveolar lavage ($n=3$).

Enzyme Requirement for Conversion of Reconstituted PL+SP-A+SP-B to Light Subtype

The presence of DFP (10mM), a specific and irreversible inhibitor of serine active enzymes, in the cycling mix inhibited generation of light subtype on cycling PL+SP-A+SP-B with convertase ($n=6$) (Fig. 5). Similarly, minimal light subtype generation occurred on cycling PL+SP-A+SP-B with boiled convertase ($n=4$; Table 1). Addition of Orlistat (50 μM), a general inhibitor of mammalian lipases, to the cycling mixture also inhibited conversion of PL+SP-A+SP-B to light subtype ($n=3$; Table

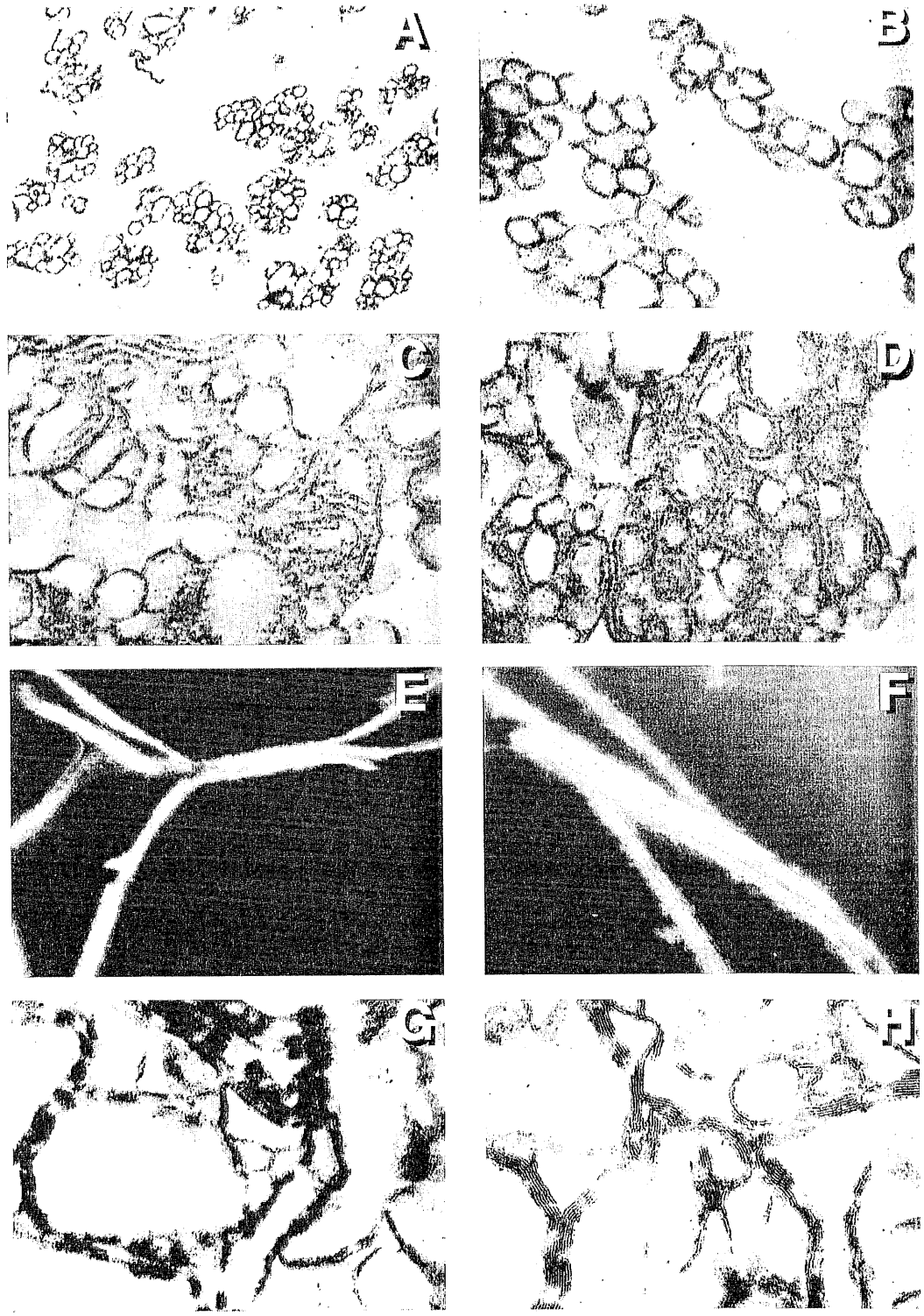

FIG. 1. Ultrastructure of various surfactants before cycling. Liposomes of DPPC:PG, 7:3 w/w were prepared by the dialysis-detergent method (panel A, magnification × 12,000). This procedure resulted in the formation of bilayered vesicles ~150 nm in size (panel B, magnification × 50,000). Addition of SP-A (5% of phospholipid w/w) resulted in formation of larger multilamellar vesicles and some multibilayered vesicles (panels C and D, magnification × 75,000). Moreover, electron dense material was present between the membrane bilayers and also dispersed between the vesicles. The lipids were arranged in sheets of parallel membranes in the presence of SP-B (0.02% of phospholipid w/w) (panels E, magnification × 75,000, and F, magnification × 150,000). Addition of both SP-A and SP-B resulted in formation of large multilamellar vesicles (panel G, magnification × 75,000), with some areas showing formation of a latticed structure (panel H, magnification × 80,000).

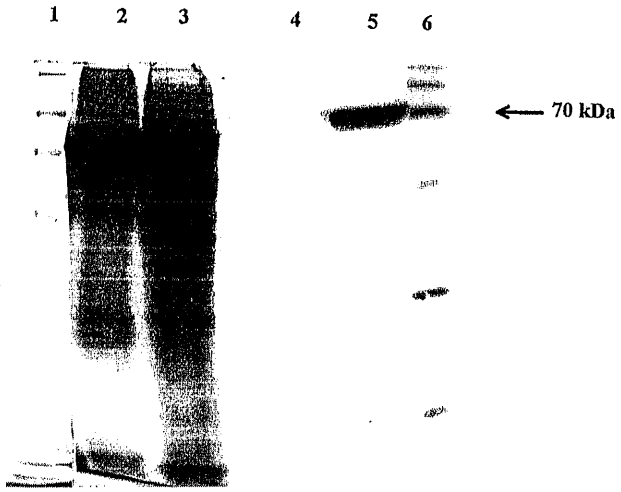

FIG. 2. SDS-PAGE and autoradiography of alveolar lavage and Concanavalin-A (Con-A) purified proteins. Lane 1- Novex Mr standards, Lane 2 - 20 μg of the acetone precipitated protein obtained from alveolar lavage which had previously been incubated with 0.11Ci/ml [^{3}H]-DFP, Lane 3-24 μg of [^{3}H]-DFP labeled protein from Con-A yield. Lanes 4 and 5 show autoradiography of the SDS-PAGE gels, Lane 6- [^{14}C] radiolabeled Mr standards. Several proteins can be resolved in alveolar lavage/Con-A yield by SDS-PAGE and staining with Coomassie Blue (Lanes 2 and 3, respectively). However, only the 72 kD protein demonstrates binding with [^{3}H]-DFP (Lanes 4 and 5). Con-A chromatography increases the yield of the 72 kD protein.

1). Therefore, conversion of reconstituted PL+SP-A+SP-B to light subtype on cycling was significantly increased by esterase or lipase activity.

Cycling with Phospholipases

We determined the effect of cycling natural heavy substrate with various phospholipases which are known to split specific bonds in phospholipids (22). Cycling with phospholipases A_2, B, and C, produced a lipolytic effect in which the heavy and light peaks were not seen, instead there was a broad band of radioactivity in the light buoyant density range. In contrast, cycling with phospholipase D produced typical heavy and light peaks after cycling with natural heavy substrate (Fig. 6). Next, we cycled reconstituted surfactant, which contains only DPPC and PG as the phospholipids, with phospholipase D and found conversion to light subtype (Fig. 6). As phospholipase D hydrolyzes the ester linkage between the base and phosphatidic acid in phospholipids, conversion of reconstituted heavy to light subtype with cycling may require splitting of choline from DPPC or glycerol from PG.

DISCUSSION

Cycling nascent surfactant reproduces the intra-alveolar metabolism of surfactant (5-7). The requirements for conversion of tubular myelin to small vesicles

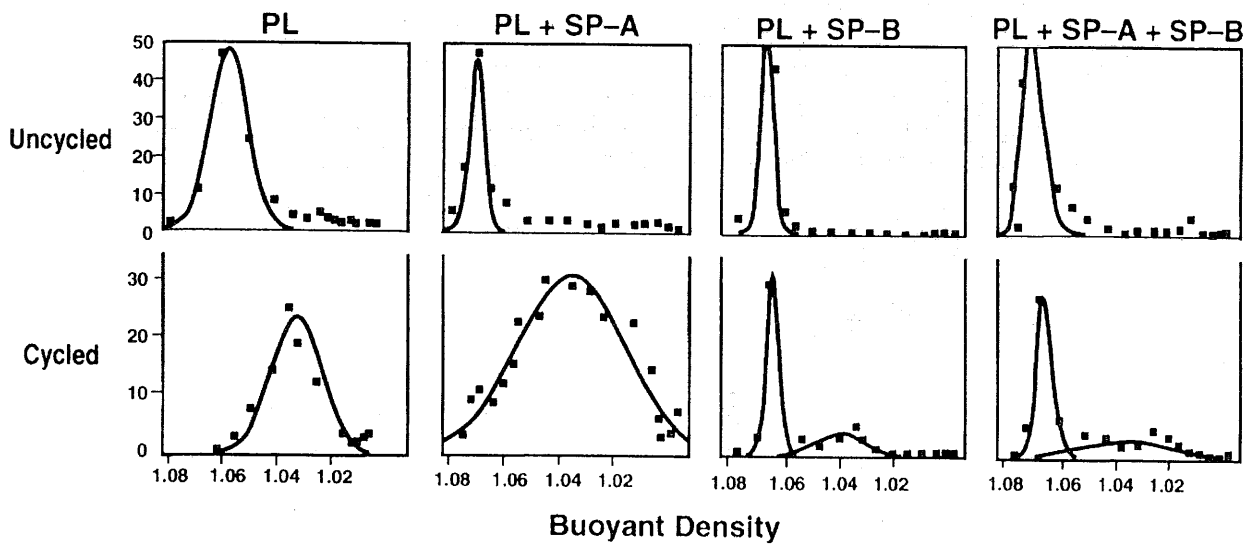

FIG. 3. Profiles of radiolabeled reconstituted surfactants containing phospholipids (PL; DPPC:PG 7:3 w/w), phospholipids and SP-A (PL+SP-A; SP-A = 5% PL, w/w), phospholipids and SP-B (PL+SP-B; SP-B = 0.02% PL, w/w), and phospholipids with both SP-A and SP-B (PL+SP-A+SP-B). Each point represents radioactivity in a fraction as a percentage of total gradient activity. Best-fit computer estimates of subtype distributions are shown. The buoyant density (g/ml) is represented in the abscissa. Profiles depicted are representative of 4 (uncycled) and 6 (cycled) experiments with each surfactant (~100 μg per cycling tube). Top panel: Uncycled surfactants. Lower panel: Surfactants cycled for 4 h. The surfactants were sedimented to equilibrium through continuous sucrose gradients (0.1 to 0.9 M). The buoyant densities of uncycled surfactants were in the heavy to ultraheavy range (1.058 to 1.074 g/ml). PL and PL+SP+A showed conversion to light subtype on cycling, but conversion of PL+SP-B or PL+SP-A+SP-B was significantly lower than that observed with PL or PL+SP-A ($p<0.001$ for each). The conversion to light subtype was significantly decreased by addition of SP-B to phospholipids.

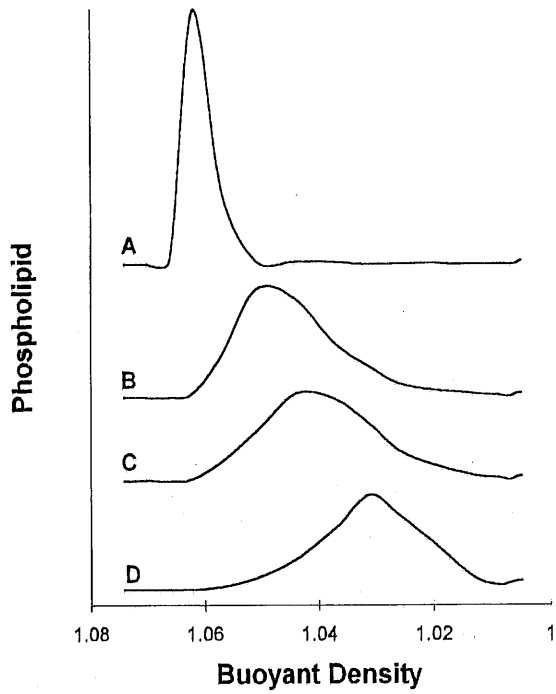

FIG. 4. Profile of light subtype generation on cycling phospholipids for varying duration. A binary mixture of radiolabeled phospholipids (DPPC:PG 7:3 w/w) was cycled for 0, 1, 2 , and 4 h (graphs A to D, respectively). Each cycling tube contained ~100 μg of phospholipid. The buoyant density (x-axis) is plotted against percent of total gradient radioactivity (y-axis). Each phospholipid mixture was sedimented to equilibrium through continuous sucrose gradients. The peak buoyant density of the phospholipid mixture before cycling was 1.057 g/ml (graph A), corresponding with heavy subtype of surfactant. After cycling for 1, 2 and 4 h there was a progressive shift in buoyant density with peaks at 1.046, 1.038, and 1.030 g/ml (graphs B to D, respectively). Therefore, a DPPC:PG mixture does not require enzyme activity for conversion to light subtype.

include cyclic changes in the surface area, temperature of 37°C, and the activity of convertase (23). In the present study, we found that a reconstituted surfactant containing synthetic phospholipids and purified SP-A and SP-B had surface activity, ultrastructure, buoyant density, and cycling behavior resembling natural surfactant (Figs. 3 and 5). The buoyant densities of the light peaks on cycling reconstituted surfactant were somewhat higher than those reported after cycling natural surfactant, but this is probably because of the higher buoyant density of the reconstituted surfactant before cycling. We also found that both natural and reconstituted surfactant converted to light subtype on cycling with phospholipase D. As reconstituted surfactant contained only DPPC and PG, our findings suggest that hydrolysis of the ester linkage between choline and/or glycerol and phosphatidic acid are at least partly responsible for conversion to light subtype during cycling. These findings serve as a first step toward elucidating the mechanism of conversion of heavy to light subtype with cycling.

To examine the influence of surfactant apoproteins on cycling properties of phospholipids, we explored the influence of each component of reconstituted surfactant on conversion to light subtype. We found, in contrast to Veldhuizen and co-workers (24), that a binary phospholipid mixture (DPPC+PG), devoid of SPs, showed partial conversion to light density forms when cycled in the absence of convertase (Fig. 3). Moreover, the conversion increased with increasing duration of cycling (Fig. 4). The disparity in our results may be due to the fact that Veldhuizen and co-workers prepared liposomes by sonication, and we confirmed that the intrinsically unstable small unilamellar vesicles prepared by this technique (21) did not change their buoyant density on cycling. Also, Veldhuizen and co-workers (24) performed cycling with a greater quantity of phospholipid and in the absence of divalent cations in the cycling mixture, both of which can influence the results (7).

The conversion of phospholipids to light subtypes was decreased by addition of SP-B but not SP-A (Fig. 3). Interestingly, the amount of SP-B in PL+SP-A+SP-B (SP-B = 0.02 % of PL w/w) was much lower than concentrations previously used to reconstitute surfactant (9-11); the amounts of SP-B that induce lipid mixing or facilitate phospholipid insertion into a monolayer in vitro; or SP-B concentrations in lamellar bodies (25-27). Addition of SP-B altered the buoyant density, cycling properties, and morphology of the phospholipids. This difference was not due to the use of proteins from patients with alveolar proteinosis as PL+SP-A+SP-B prepared from proteins derived from mice also showed similar buoyant density and cycling properties. Therefore in addition to its known functions (28), another role of SP-

TABLE 1

Conversion of Reconstituted Surfactant[a] to Light Subtype on Cycling[b]

	% L
Uncycled	4.1 ± 1.6
Cycled with AL buffer	14.3 ± 3.8
Cycled with convertase	58.2 ± 6.7
Cycled with boiled convertase	18.1 ± 4.0
Cycled with convertase + DFP (10 mM)	7.9 ± 2.4
Cycled with convertase + Orlistat (50 μM)	16.3 ± 5.1

Note. Mean ± SE of ≥ 10 experiments for surfactant which was uncycled, cycled, and cycled with convertase, and 4-6 experiments with inhibitors.

[a] DPPC:PG (7:3 w/w) + SP-A (5% w/w) + SP-B (0.02% w/w)

[b] Approximately 100 μg of [^{14}C]-labeled reconstituted surfactant was either not cycled, cycled for 4 h with AL buffer, or cycled for 4 h with the indicated enzyme and inhibitor. The products were separated on sucrose gradients and the radioactivity in each fraction plotted as a percentage of the total radioactivity in the gradient. The peaks of radioactivity were integrated to determine the amounts of phospholipid in each peak. Conversion to light subtype was calculated as the proportion of total activity (%L) that was found under the light peak.

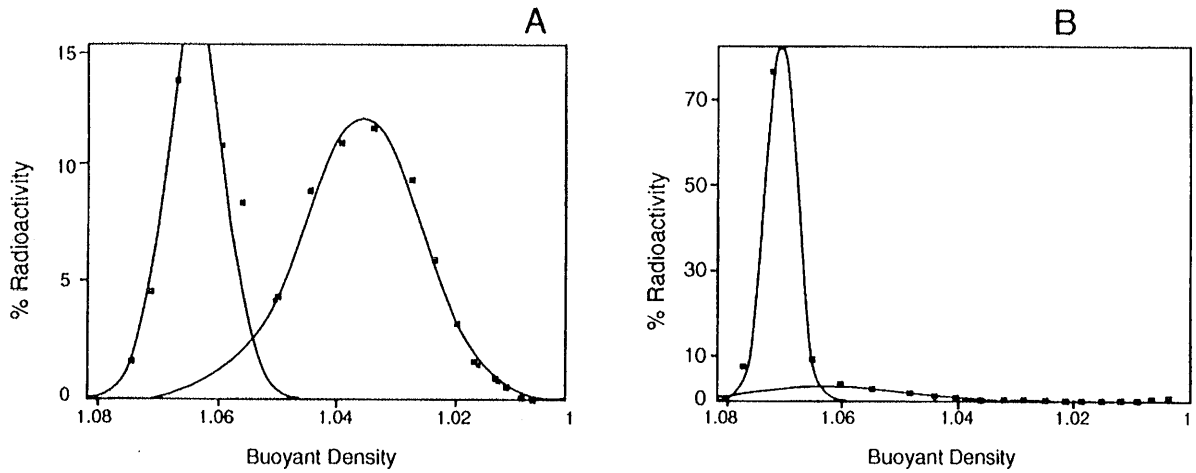

FIG. 5. Effect of cycling reconstituted surfactant with convertase. Reconstituted surfactants containing phospholipids, SP-A (5% of PL, w/w) and SP-B (0.02% of PL, w/w) (approximately 100 μg/cycling tube) were cycled with convertase (~30 μg of protein/cycling tube) at 37°C for 4 h. A representative profile of 8 separate experiments is shown in panel A. The results of cycling a similar surfactant and convertase when pre-incubated with DFP (10 mM final concentration) at 37°C for 30 min before cycling are shown in panel B. The surfactants were processed as depicted in Figure 3. The conversion of PL+SP-A+SP-B to light subtype on cycling with convertase was inhibited by DFP.

B may be to preserve surfactant in the surface active heavy density form within alveoli, and the concentrations of SP-B required to decrease conversion of surfactant may be much lower than those required to fulfill some of its other functions.

Cycling with reconstituted surfactant (PL+SP-A+SP-B) resulted in minimal light subtype generation, and enzyme activity was required for conversion of heavy to light subtype. We used a partially purified convertase preparation (Con-A yield) because the yield of pure convertase from alveolar lavage is too small for use in routine cycling experiments. There are more than 20 proteins in Con-A yield; however, several lines of evidence indicate that the single 72 kD DFP-binding protein, which we identify with convertase, is responsible for conversion of heavy to light subtype on cycling. First, the DFP-binding band has been purified and found to be a carboxylesterase (8). Second, there is a

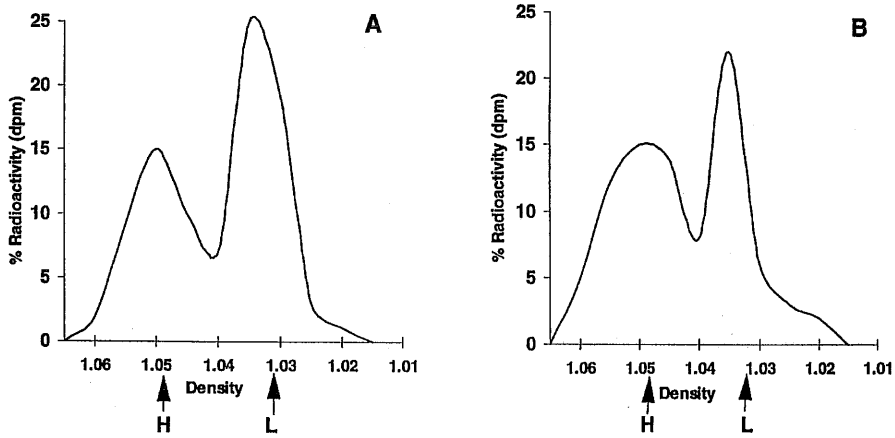

FIG. 6. Effect of cycling radiolabeled natural heavy substrate or radiolabeled reconstituted surfactant with peanut phospholipase D (5 units/ml). The endogenous convertase activity of natural heavy substrate was inhibited by pre-incubation with cold DFP (10 mM). The profiles of distribution of radioactivity as seen after centrifugation to equilibrium through continuous sucrose gradients are shown. Arrows represent heavy and light peaks. Panel A: Cycling natural heavy substrate with Phospholipase D (n = 6) produced conversion (45±8.1%) to light subtype (mean buoyant density 1.033 g/ml) with some phospholipid remaining at a heavy buoyant density (mean 1.05 g/ml). Panel B: A similar profile was obtained on cycling reconstituted surfactant with Phospholipase D (n = 6) with 39±4.8% of the phospholipid converting to light subtype with a mean buoyant density of 1.035 g/ml. Cycling in the absence of enzyme revealed minimal (<15%) light subtype generation with either surfactant (see Figure 3 for comparison). Similar results were obtained on cycling with cabbage phospholipase D. Therefore, phospholipase D significantly increased ($p<0.01$) conversion of natural and reconstituted heavy surfactant to light subtype on cycling.

single DFP-inhibitable esterase in Con-A yield (8). Third, inhibition of conversion of PL+SP-A+SP-B to light subtype on cycling by DFP and Orlistat verify that reconstituted surfactant behaves similarly to natural surfactant (29) on cycling with these inhibitors (Fig. 5, Table 1), and provides evidence that esterase/lipase activity is required for conversion of PL+SP-A+SP-B to light subtype on cycling. Further experiments showed that the typical profile of conversion to heavy and light subtypes was observed on cycling with phospholipase D but not with phospholipases A_2, B or C. Phospholipase D hydrolyzes the ester linkage between the base and phosphatidic acid in phospholipids (22). As reconstituted surfactant contained only DPPC and PG, conversion of PL+SP-A+SP-B to light subtype on cycling with phospholipase D suggests that split of the choline and/or glycerol linkage with phosphatidic acid may be at least one mechanism for conversion to light subtype during cycling. Whether convertase exhibits phospholipase D like action needs to be explored in future experiments.

Reconstituted surfactants are not fully representative of natural surfactant. For example, PL+SP-A+SP-B contains only DPPC and PG whereas natural surfactant is a complex mixture of phospholipids. Moreover, PL+SP-A+SP-B does not contain SP-C which is known to facilitate formation of a surface film (30). However, the role of SP-C in surfactant metabolism is unclear, it cannot substitute for SP-B in forming tubular myelin, and it does not alter the structure of PL+SP-A+SP-B (10). Studies with reconstituted surfactants containing more complex phospholipid mixtures and SP-C are planned but these studies are tedious because each surfactant needs reformulation to achieve optimal cycling properties.

In summary, very small quantities of SP-B in a phospholipid mixture decreased conversion to light density forms on cycling. Reconstituted surfactants containing phospholipids and both SP-A and SP-B required convertase activity for conversion to light subtype, an effect similar to that observed with native tubular myelin. The typical profile of heavy and light subtype generation was also observed on cycling natural or reconstituted surfactant with phospholipase D. In conclusion, the addition of SP-B to phospholipids decreases their conversion to light subtypes, and phospholipids combined with SP-A and SP-B require esterase/phospholipase D activity for transformation to light subtype on cycling.

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