Surfactant phospholipid changes after antigen challenge: a role for phosphatidylylglycerol in dysfunction

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Although the importance of surfactant in the physiology of conducting airways was first reported approximately 30 years ago, acceptance of this principle has occurred only recently (26). Multiple structural and animal models have described the importance of a surfactant film to provide surface tension lowering and maintenance of airflow within the conducting airways (12). The relative role of surfactant dysfunction in asthma has been implicated by animal and human models of antigen challenge and by several small clinical trials that suggest surfactant replacement therapy may improve airflow obstruction in acute asthma (2, 24, 25).

Two separate groups have reported surfactant dysfunction in humans after endobronchial antigen challenge (21, 22). Each group identified significant protein changes in the surfactant pellet, which correlated with surfactant function, and concluded that the principal mechanism of surfactant dysfunction was leak of serum protein. One subsequent study of surfactant phospholipid composition after antigen challenge demonstrated significant changes in the fatty acid distribution, consistent with leak of serum phospholipids, lipoproteins, and fatty acids (17). However, based on ex vivo studies examining protein inhibition of surfactant function, we speculated that surfactant abnormalities in asthma are not sufficiently explained by the reported increases in protein (30, 37). We hypothesized that the surfactant dysfunction of asthma is more likely to be a multifactorial process in which protein leak is a likely contributor but also includes direct inflammation-mediated injury of surfactant.

Our group has reported increased secretory phospholipase A2 (sPLA2) activity after antigen challenge (5, 7) and demonstrated that sPLA2 hydrolyze surfactant-associated phospholipids in vitro (20). In subsequent studies, our data have suggested that sPLA2-mediated surfactant dysfunction is multifactorial and can be dependent on hydrolysis of phosphatidylglycerol (PG) as well as the generation of lysophospholipids (19). Therefore, we hypothesized that changes in surfactant phospholipids may result from inflammation during asthma and that these changes could generate surfactant dysfunction.

To further test this hypothesis, we studied a cohort of mild asthmatics who underwent endobronchial antigen challenge and bronchoalveolar lavage (BAL). The cellular response, protein leak, and surfactant in the BAL fluid (BALF) was compared with BALF from saline-challenged lobes of the same asthmatics. The results demonstrate significant alterations in surfactant function and composition including a shift of large functional surfactant aggregates (LA) to small dysfunctional aggregates (SA), a decrease in PG, and an increase in the ratio of phosphatidylcholine (PC) to PG (PC/PG). These end points are indicative of direct surfactant injury and correlate more strongly with surfactant dysfunction than does protein leak or eosinophil influx. In addition, BAL sPLA2 activity increased, which may explain the PG depletion. Some of the results of these studies have been previously reported in the form of an abstract (18).

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**METHODS**

**Patient Selection**

Subjects with atopic asthma (n = 13) were studied. Each asthmatic met criteria of reversible airflow obstruction demonstrated by an increase in forced expiratory volume in 1 s (FEV1) of ≥15% following β-agonist inhalation, or a methacholine pharmacological challenge test that results in a 20% decrease in FEV1 (PC20) at a dose of <8 mg/ml, and episodic symptoms of including cough, dyspnea, and wheezing (1). Subjects with significant cigarette smoking exposure (>5 pack yr) were excluded. Anti-inflammatory medications including inhaled and systemic corticosteroids, cromones, and leukotriene modifiers were withheld for at least 6 wk before study initiation. Inhaled β-agonists (except salmeterol) were withheld for 6 h, and salmeterol and theophylline were withheld for 24 h before each study. Before enrollment, all subjects completed an informed consent, which was approved by our Institutional Review Board.

**Antigen Challenge**

To determine the dose used for subsegmental endobronchial challenge, inhaled antigen challenges were performed in each asthmatic subject using cat dander (Felis domesticus), dust mite (Dermatophagoides farinae), short ragweed (Greer Laboratories, Lenoir, NC), or standardized cat hair (ALK Laboratories, Wallingford, CT) based on results of prior skin testing. Antigens were administered via a continuous LC Jet+ nebulizer (PARI Respiratory Equipment, Richmond, VA) over 2 min of tidal breathing. The FEV1 was measured at 5-min intervals, and the antigen concentration increased every 15 min until a 20% drop in FEV1 or the highest concentration (1:10) was achieved. Subjects were monitored for a minimum of 10 h following antigen challenge, including determination of late asthmatic response (LAR). All subjects experienced an LAR defined as a drop in FEV1 of at least 15%, which persisted for >30 min. We used 5 ml of a 1:10 dilution of the concentration resulting in a 20% fall in FEV1 for the segmental challenge.

**Endobronchial Antigen/Saline Challenge**

Bronchoscopy for challenge was performed on day 1 with segmental challenge in either the lingula or right middle lobe using 5 ml of antigen solution described above (and the bronchoscope channel flushed with 5 ml of saline) and 10 ml of saline on the contralateral side. Bronchoscopy was repeated 24 h later (day 2), and bilateral BALs were performed.

**BAL**

BAL was performed 24 h following endobronchial application of antigen to a single segment of one lung and saline to a single segment of the opposite lung. Fiber-optic bronchoscopy was performed using nebulized and topical Xylocaine with benzodiazepine sedation. A single inhalation of isoproterenol (0.131 mg) or albuterol (0.09 mg) was administered via metered-dose inhaler, and then the bronchoscope was introduced transnasally into the lower airways. BAL was performed with three 50-ml aliquots (150 ml total volume) of sterile saline, and BALF was kept on ice until processed (30 min).

**Cell Isolation**

The total BALF was measured for volume and centrifuged at 200 g for 15 min. The supernatant was removed and further processed for isolation of surfactant (below). The resulting cell pellet was washed three times with PBS and resuspended in PBS containing 0.1% gelatin. Total cell count and viability were determined by hemacytometer and trypan blue exclusion. Differential cell counts were determined from Cytospin slides (Thermo Shandon, Pittsburg, PA) using Leukostat stain.

**Isolation of Surfactant**

BALF supernatant (cell free) was centrifuged at 45,000 g for 1 h. Aliquots of the resulting supernatant were stored at −70°C. The surfactant pellet was washed three times with 100 ml of normal saline (pH 7.4) and resuspended in a known volume of normal saline. Aliquots were stored at −70°C. LA were defined as those in the pellet after ultracentrifugation, whereas SA were defined as the surfactant remaining in the supernatant (35). The phospholipid content of LA and SA was measured by quantitation of lipid phosphorous using the methods of Bartlett (3). “Total” surfactant recovered represents the sum of LA and SA.

**Surfactant Function**

A pulsating bubble surfactometer (Electroanemics, Amherst, NY) was used to measure surface tension lowering activity (11). Surfactant from the LA fraction (40 μl) was used at a final concentration of 1.0 mg phospholipid/ml in buffered saline containing 5 mM CaCl2 and was analyzed at 20 cycles/min for 10 min at 37°C in the surfactometer as described previously (20). The results are reported as Ymin, which represents the minimum surface tension achieved over the 10-min period.

**Protein**

The protein content of the cell-free BALF supernatant and the surfactant pellet was measured by the bicinchoninic acid method (Pierce, Rockford, IL), with BSA as standards.

**Phospholipid Composition**

The phospholipid fraction of samples was extracted via the methods of Bligh and Dyer (4). After extraction, the organic phase was dried under N2, resuspended in chloroform, and analyzed for phospholipid concentration (3). Phospholipid composition of LA was determined by separation of individual phospholipids with high-performance liquid chromatography (HPLC) using a C-18 Kromasil column (Drachrom, Greensboro, NC) and a dual solvent system gradient from 100% Mobile Phase A (CHCl3/MeOH/NH4OH, 80:19.5:0.5) ramped to a maximum of 25% Mobile Phase B (MeOH/H2O/NH4OH, 80:19.5:0.5). Quantitation of individual phospholipid peaks from the HPLC eluent was performed with an evaporative light scatter detector (ELSD; SEDERE, Alfortville, France). Retention times and binomial response characteristics of individual phospholipids [PG, PC, phosphatidylinositol (PI), phosphatidyethanolamine, sphingolipids] and lysophospholipids [lysophosphatidylglycerol (LPG), lysophosphatidylcholine (LPC)] were defined with commercially available standards (Avanti Polar Lipids, Alabaster, AL). This sensitive method measures individual phospholipids in the range of 0.1–4.0 nmol and requires a minimum surfactant sample size of 12 nmol. To calculate absolute phospholipid concentrations, a fixed concentration of phosphatidylbutanol was added to a known concentration of sample after lipid extraction to serve as an internal standard.

**Phospholipase (PLA2) Activity**

Supernatant from each BALF (475 μl) was incubated with Surfanta (Ross Laboratories, St. Louis, MO), which was labeled with either [3H]palmitate-dipalmitoylphosphatidylcholine (DPPC, 4.4 × 10^−9 nmol/μl, 0.1 μCi/μl) or [3H]oleate-PG (0.026 nmol lipid/μl, 0.004 μCi/μl) using repetitive vortexing. The [3H]PC was purchased from New England Nuclear (Boston, MA). The [3H]PG was prepared by TLC purification of PG from membranes of [3H]oleate-labeled Escherichia coli using previously reported methods (23, 33). The phospholipid concentration of the final mixture (500 μl) was 1.0 mg/ml (583.4 nmol of phospholipid/sample). Reaction mixtures were supplemented with CaCl2 (5.0 mM) and incubated at 37°C for 4 h. Lipid extraction was used to stop hydrolysis (4), and the phospholipid...
Table 1. Antigen challenge in total asthmatic population

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Antigen</th>
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<tbody>
<tr>
<td>BAL cells, × 10⁶/ml</td>
<td>34.5 ± 5.0</td>
<td>124.3 ± 39.2*</td>
</tr>
<tr>
<td>Total cells</td>
<td>1.2 ± 0.5</td>
<td>67.8 ± 26.5*</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>248.3 ± 108.5</td>
<td>1207.9 ± 597.7</td>
</tr>
<tr>
<td>Surfactant pellet, µg/mg phospholipid</td>
<td>219.4 ± 12.9</td>
<td>315.1 ± 37.5*</td>
</tr>
</tbody>
</table>

Results are means ± SE. BAL, bronchoalveolar lavage; LA, large aggregate; SA, small aggregate; PC, phosphatidylcholine; PG, phosphatidylglycerol. *P < 0.05 comparison of saline and antigen challenged observations by ANOVA.

and free fatty acid fractions from the organic phase of the lipid extract were measured as previously reported (20). Hydrolysis is expressed as the percentage of the total radioactivity in the free fatty acid fraction.

Statistics

Data represent means ± SE. Analysis of variance was used to determine correlations between changes in the saline-challenged and antigen-challenged samples. Statistical significance for comparison of subjects with dysfunctional surfactant after antigen challenge and those with normal surfactant function after antigen challenge was determined by paired and unpaired Student’s t-tests and Spearman rank correlation.

RESULTS

Antigen Challenge in Asthmatic Population

The differences in BALF cellular response, protein, and surfactant between asthmatics after undergoing segmental endobronchial antigen challenge 24 h earlier and a paired segmental challenge with saline are summarized in Table 1. There was no difference in the volume of BALF recovered between saline-challenged and antigen-challenged samples (54.8 vs. 52.8%, n = 13).

Antigen challenge resulted in increased total BAL cells relative to the paired saline-challenged asthmatic samples (124.3 × 10⁴ vs. 34.5 × 10⁴ cells/ml, P < 0.05). All individual cell types analyzed (eosinophils, neutrophils, lymphocytes, and alveolar macrophages) were greater in the antigen-challenged samples, but only the eosinophils were increased significantly (67.8 × 10⁴ vs. 1.2 × 10⁵ cells/ml, P < 0.03). For the entire subject group, eosinophils were increased in 12 of 13 patients, and these cellular changes are consistent with changes reported by Shaver et al. (31) after subsegmental endobronchial antigen challenge of asthma patients.

Increased total BALF protein has been hypothesized by others to be the major mechanism of surfactant dysfunction after antigen challenge (21, 22). Because we measured pulmonary surfactant function using only the phospholipid from the LA fraction in the surfactant pellet resuspended in sterile saline, we measured the protein content of the LA pellet fraction separately from the protein in the BALF supernate. The concentration of protein in the surfactant pellet has been calculated as µg of protein per mg of phospholipids, corresponding to functional studies performed at a phospholipid concentration of 1 mg/ml. As seen in Table 1, protein in the surfactant LA pellet of the antigen-challenged samples was ~50% greater than the saline-challenged samples (315.1 vs. 219.4 µg/mg phospholipids, P < 0.05). BALF supernatant protein was highly variable, such that the increase in the antigen-challenged samples did not reach statistical significance. Thus we confirmed that BALF protein concentrations increased in response to antigen challenge and notably extended that observation to the surfactant LA pellet where function is measured.

Surfactant function was measured as the γmin in the LA pellet using a pulsating bubble surfactometer (Table 1). The antigen-challenged samples demonstrated significantly higher γmin than the paired saline-challenged samples (10.1 vs. 2.4 mN/m, P < 0.05). Table 1 also details the BALF phospholipid recovery, distribution between the LA of the ultracentrifuged pellet and the SA of the remaining supernatant, plus the calculated LA/SA ratio. Total phospholipid recovery (LA + SA) was not significantly different between antigen-challenged and saline-challenged samples. However, the LA/SA ratio was significantly decreased in the antigen-challenged BALFs (2.9 vs. 4.4, P < 0.05). This change in distribution represents a combined decrease in phospholipids in the LA fraction and an increase in phospholipids in the SA fraction, neither of which reached statistical significance separately. In the LA fraction, the two most abundant phospholipids, PC and PG, were decreased but did not reach statistical significance.

Spectrum of Surface Tension-Lowering Activity After Antigen Challenge

Despite a consistent inflammatory response to antigen challenge with increased cells and protein in 12 of 13 patients, surfactant dysfunction as measured by an increased γmin was seen in only 6 of 13 subjects after antigen-challenge between subjects (Fig. 1). The increase in γmin in those subjects with

![Fig. 1. Surfactant function. Asthmatics (n = 13), after endobronchial challenge with saline and antigen, underwent bronchoalveolar lavage (BAL). Surfactant pellets were isolated from BAL and resuspended in buffered saline (1.0 mg phospholipid/ml) with 5.0 mM CaCl₂. Minimum surface tension (γmin) was measured with a pulsating bubble surfactometer over 10 min at 37°C. The data represent means ± SE. The dotted line in the antigen-challenged asthmatic results is used to define “dysfunctional surfactant” (>8.0 mN/m) and “normal surfactant” (<8.0 mN/m). *P < 0.05 comparison of saline- and antigen-challenged observations by ANOVA.](http://ajplung.physiology.org/)
“dysfunctional surfactant” was highly significant (19.6 vs. 2.4 mN/m, \( P < 0.001 \) vs. paired saline sample). The remaining seven subjects had no significant change or “normal surfactant” (1.9 vs. 2.4 mN/m). The absolute value of \( \gamma_{\text{min}} \) to differentiate between dysfunctional surfactant and normal surfactant function subsets was 8.0 mN/m (Fig. 1), a value determined using the upper 95% confidence limit for the measured \( \gamma_{\text{min}} \) in the saline-challenged samples (7.7 mN/m). To test for potential mechanisms that explain surfactant dysfunction, the end points from Table 1 were re-examined with separation of the dysfunctional and normal surfactant asthmatic subgroups.

Comparisons Between the Normal and Dysfunctional Surfactant Groups

**BAL cells.** With antigen challenge, total cells increased significantly within the dysfunctional surfactant subset (192.8 \( \times 10^4 \) vs. 28.2 \( \times 10^4 \) cells/ml, \( P < 0.05 \)), shown in Table 2. This intense cellular infiltrate was predominantly characterized by significant increases in eosinophils (114.3 vs. 1.3, \( P < 0.05 \)). Lymphocytes and alveolar macrophages also increased in the dysfunctional surfactant subset after antigen challenge, but the differences did not reach statistical significance. In the normal surfactant group, there was an increase in total cells and eosinophils (21.3 \( \pm 12.5 \) vs. 1.2 \( \pm 0.3 \)), but neither change was statistically significant. There were no differences in BALF volume recovery in the dysfunctional and normal surfactant subgroups (not shown).

Because eosinophils have been recently implicated as being important in surfactant dysfunction after antigen challenge, we performed correlation analysis between total eosinophil count and \( \gamma_{\text{min}} \) (Fig. 2A) (21). Although the correlation was statistically significant (\( R = 0.54, P < 0.05 \)), the data were notable for two subjects with significantly increased \( \gamma_{\text{min}} \) without a significant change in eosinophils and two subjects with significantly increased eosinophils without a significant change in \( \gamma_{\text{min}} \).

**Protein.** Consistent with the previously discussed hypothesized importance of protein leak in surfactant dysfunction after antigen challenge, protein in the surfactant pellet increased significantly after antigen challenge within the dysfunctional surfactant subset (376.8 vs. 197.6 \( \mu \)g/mg phospholipid, \( P < 0.05 \)), as shown in Table 2. Protein did not change significantly in the normal surfactant subset group. The correlation of the protein increase on surfactant function after antigen challenge was then examined (Fig. 2B). Similar to the results seen with eosinophils, there was a significant correlation (\( R = 0.61, P < 0.03 \)), which was slightly stronger than seen with eosinophils, but the \( R \) value was heavily influenced by two subjects with very high protein levels. A similar degree of correlation was seen when we compared the BAL supernatant protein with \( \gamma_{\text{min}} \) (data not shown).

**Surfactant phospholipid.** The LA/SA ratio was significantly decreased in the dysfunctional surfactant group after antigen challenge (1.3 vs. 4.9, \( P < 0.001 \)), but not in the normal

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**Table 2. Antigen challenge analyzed according to level of surfactant dysfunction**

<table>
<thead>
<tr>
<th></th>
<th>Normal Surfactant (n = 7)</th>
<th>Dysfunctional Surfactant (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>BAL cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils, ( \times 10^4 )/ml</td>
<td>1.2( \pm 0.3 )</td>
<td>21.3( \pm 12.5 )</td>
</tr>
<tr>
<td>BAL protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surfactant pellet, ( \mu )g/mg phospholipid</td>
<td>238.4( \pm 20.3 )</td>
<td>262.3( \pm 22.4 )</td>
</tr>
<tr>
<td>Surface Tension, mN/m</td>
<td>2.4( \pm 0.8 )</td>
<td>1.9( \pm 0.8 )</td>
</tr>
<tr>
<td>Total phospholipid recovery, ( \mu )g/ml</td>
<td>36.4( \pm 4.7 )</td>
<td>25.9( \pm 4.1 )</td>
</tr>
<tr>
<td>LA recovery, ( \mu )g/ml</td>
<td>28.1( \pm 3.1 )</td>
<td>20.2( \pm 3.2 )</td>
</tr>
<tr>
<td>SA recovery, ( \mu )g/ml</td>
<td>8.2( \pm 1.5 )</td>
<td>5.7( \pm 1.4 )</td>
</tr>
<tr>
<td>LA/SA ratio</td>
<td>3.9( \pm 0.5 )</td>
<td>4.3( \pm 0.6 )</td>
</tr>
<tr>
<td>PC, ( \mu )g/ml</td>
<td>22.6( \pm 2.3 )</td>
<td>15.0( \pm 2.8 )</td>
</tr>
<tr>
<td>PG, ( \mu )g/ml</td>
<td>3.4( \pm 0.5 )</td>
<td>3.2( \pm 0.5 )</td>
</tr>
<tr>
<td>PC/PG ratio</td>
<td>6.8( \pm 0.4 )</td>
<td>4.8( \pm 0.6 )</td>
</tr>
<tr>
<td>( \Delta ) PC/PG, antigen/saline</td>
<td>0.70( \pm 0.08 )</td>
<td></td>
</tr>
</tbody>
</table>

Results are means \( \pm \) SE. \( *P < 0.05 \) comparison of paired antigen- vs. saline-challenge in the dysfunctional surfactant group; \( †P < 0.05 \) comparison of dysfunctional to normal function groups after antigen challenge.

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Fig. 2. Correlation of eosinophils and protein with surfactant function. The recovery of eosinophils (A) and the concentration of surfactant pellet protein (B) are plotted against \( \gamma_{\text{min}} \). The subset of subjects with dysfunctional surfactant (■) is defined as \( \gamma_{\text{min}} \geq 8 \) mN/m after antigen challenge, whereas those subjects with normal surfactant (○) are <8 mN/m.
surfactant subset (Table 2). Although the LA was reduced after antigen challenge, the difference did not achieve statistical significance in either subset. SA increased significantly in the dysfunctional surfactant subset after antigen challenge (11.9 vs. 5.3 µg/ml, \( P < 0.005 \)) but did not increase in the normal surfactant subset.

Correlation between the decrease in LA/SA ratio with the increase in \( \gamma_{\text{min}} \) was higher than seen for either eosinophils or protein (\( R = -0.73, P < 0.005 \), Fig. 3), which suggests that changes that affect the distribution of surfactant phospholipid between LA and SA after antigen challenge may provide insight into the mechanism of surfactant dysfunction.

**Phospholipid composition.** As our primary end point of significance was surfactant function, which was measured on the LA fraction, we primarily focused our analysis on the phospholipid composition from the LA surfactant pellets using HPLC/ELSD. Although the phospholipid content of the SA fraction was not a contributor to surfactant function in our approach, the SA fraction composition was analyzed to completely determine the fate of any changes identified in the LA analysis.

A significant decrease in PG in the LA fraction was seen after antigen challenge in the dysfunctional surfactant group (1.6 vs. 3.4 µg/ml, \( P < 0.05 \)), whereas PG in the normal surfactant subset was unchanged after antigen challenge (Table 2). The amount of PG recovered was also significantly different on direct comparison of the antigen-challenged dysfunctional surfactant subset to the antigen-challenged normal surfactant subset (1.6 vs. 3.2 µg/ml, \( P < 0.05 \)). In the total BAL (LA + SA) of the dysfunctional surfactant group, PG decreased slightly but did not reach statistical significance. In the normal surfactant subset, the total PG content increased slightly but also did not reach statistical significance. The reduced PG content in the LA fraction of the antigen-challenged samples correlated with \( \gamma_{\text{min}} \) (Fig. 4A) with an \( R \) value (\( R = -0.63 \)) that was as high or higher than the \( R \) values with protein or eosinophils.

The absolute change in an individual phospholipid such as PG typically does not explain surface tension-lowering activity of in vitro monolayers of phospholipid mixtures (10). To further investigate the PG changes as a component of the total phospholipid pool, we examined the ratio of PC to PG (Table 2). The dysfunctional surfactant group revealed significantly increased PC/PG (7.5 vs. 4.8, \( P < 0.05 \)) after antigen challenge, whereas the PC/PG was unchanged in the normal surfactant group. The increased PC/PG reflected primarily the decrease in PG, since the PC content tended to decrease in both groups. In addition, we compared the relative change in PC/PG between antigen-challenged and saline-challenged samples (calculated by dividing PC/PG of antigen by PC/PG of saline sample, or \( \Delta \text{PC/PG} \)). With this index, the difference between dysfunctional surfactant and normal surfactant subsets was highly significant (1.29 vs. 0.70, \( P = 0.001 \)). Figure 4B reveals the strong correlation between the \( \Delta \text{PC/PG} \) and LA/SA ratio (\( R = -0.68, P < 0.005 \)). The \( \Delta \text{PC/PG} \) index similarly demonstrated a strong correlation (\( R = -0.61, P < 0.005 \)) with an increase in \( \gamma_{\text{min}} \) (not shown). Similar indexes for changes in protein and eosinophils between the antigen-challenged and saline-challenged samples did not improve their respective correlation with surfactant function (data not shown).

Interestingly, a decrease in PI, an anionic phospholipid similar to PG, was also seen in the dysfunctional surfactant group vs. the normal surfactant subset after antigen challenge (0.4 vs. 0.8 µg/ml, \( P < 0.02 \), not shown).

LPC and LPG were analyzed in the LA and SA fractions of all samples. Only a few samples (pre- and postantigen) were
identified to have any LPC or LPG, and no single sample had more than a trace amount.

**Phospholipase activity.** One potential mechanism that would alter the phospholipid content of surfactant is hydrolysis of surfactant by phospholipases. sPLA₂ activity of the BALF supernatant from our samples was measured by hydrolysis of both [³H]PG-labeled and [³H]PC-labeled exogenous surfactant (Fig. 5). Hydrolysis of PG and PC were both significantly greater in the total group of antigen-challenged samples in comparison with their paired saline-challenged samples (PG: 11.6 vs. 4.4%, \( P < 0.02 \); and PC: 17.3 vs. 6.7%, \( P < 0.05 \)). The increased PLA₂ activity after antigen challenge vs. the paired saline samples approached significance in the dysfunctional surfactant subset for hydrolysis of PG (15.3 vs. 3.8, \( P = 0.08 \)) but was virtually unchanged in the normal surfactant subset. The increased PLA₂ activity for hydrolysis of PC did not reach significance for either surfactant subset, and the increased activity was approximately equal between the two subsets.

**DISCUSSION**

Our data show significant surfactant dysfunction can result when stable baseline asthmatics undergo subsegmental endobronchial antigen challenge. The surfactant dysfunction covers a spectrum of responses within the total group of asthmatics, but analysis of subjects with significant changes in surfactant function (dysfunctional) vs. those with little or no change (normal surfactant function) highlights the potential mechanisms that may produce surfactant dysfunction. Specifically, we identify a significant decrease in the PG content of surfactant after antigen challenge. This relative drop in PG correlates closely with biophysical properties of the dysfunctional surfactant, seen as an increased \( \gamma_{\min} \) and as an altered distribution of surfactant phospholipid reflected in the decreased LA/SA.

The observed changes in surfactant aggregates indicate that the mechanisms of asthma-associated surfactant dysfunction involve direct, though complex, changes in the biophysical properties of the surfactant. Shift of surfactant aggregates from LA to SA may result from enzymatic conversion (e.g., convertase) or from inflammatory processes that target surfactant (9, 36). Although the shift from LA to SA may be the end result of a variety of pathophysiological processes, the addition of serum proteins has not been identified as a mechanism for such shifts. The changes seen in our data include decreased LA after antigen challenge, with a significantly greater decrease seen in the dysfunctional surfactant subgroup than the normal surfactant function subgroup. In addition, an increase in SA is seen

![Fig. 5. Phospholipase hydrolysis of PG.](https://example.com/fig5.png)

Fig. 5. Phospholipase hydrolysis of PG. The phospholipase activity in BAL supernatant was measured by incubating exogenous surfactant labeled with [³H]oleate-PG (A) and [³H]palmitate-PC (B) for 4 h at 37°C in saline with 5 mM CaCl₂. Hydrolysis represents formation of labeled free fatty acid, which was measured using TLC and scintillation counting. The total population of asthmatics (gray bars) is shown along with the subset of subjects with dysfunctional surfactant (closed bars), which are defined as \( \gamma_{\min} \geq 8 \text{mN/m} \) after antigen challenge, whereas those subjects with normal surfactant (open bars) are <8 mN/m. Results are means ± SE. *\( P < 0.05 \) comparison of saline- and antigen-challenged observations by ANOVA.
only in the group with dysfunctional surfactant. These combined changes result in a dramatic decrease in the LA/SA ratio, which correlates well with surfactant dysfunction ($R = 0.73$, $P < 0.005$).

The alterations in phospholipid composition of the surfactant pellets provide additional evidence that asthma-associated surfactant dysfunction is caused, at least in part, by direct injury to surfactant components. The most important alteration in composition is the decrease in PG seen in the dysfunctional surfactant group. The decreased PG is the principal reason for the increase in PC/PG index, which correlates more strongly with surfactant dysfunction and decrease in LA/SA than do the less-specific markers of inflammation, protein, and eosinophils. This increase in PC/PG ratio is in contrast to other reports of a decreased PC/PG ratio in asthmatic surfactant. In those reports, an increase in PC was noted, and the increased PC was similar in its fatty acid composition to plasma lipoproteins (17), which likely reflects the phospholipid composition was measured on the entire BAL including LA and SA fractions. This explanation is further supported by our observation that the PG content of the SA fraction from our samples was not decreased. In our current study, analysis of surfactant function utilized only the LA fraction as did our correlations between function and phospholipid composition. The decreases in PC and PG in our study supports the hypothesis that antigen challenge directly causes changes in surfactant lipid composition.

As the second most abundant phospholipid in surfactant, PG performs key functions in adsorption, spreading, and repreading of the phospholipid layer (34). The interaction of PG, an anionic phospholipid, with the essential hydrophobic surfactant protein, SP-B, may be the central mechanism through which PG performs these functions (6). Interactions between SP-B and PG play important roles in maintaining the alveolar surfactant layer by enhancing the adsorption of DPPC to the surface after release from the type II pneumocytes and repreading of the monolayer after maximum compression (exhalation) (14). During maximum compression, PG promotes formation of surface-active pools of phospholipid, which remain just below the surface and are immediately available on re-expansion (inhalation). Furthermore, deficiency of PG has been reported in other models of inflammation-associated airways disease, including the respiratory distress syndromes and cystic fibrosis (13, 15, 28).

In our subjects, PG and PI are both decreased after antigen challenge in the dysfunctional surfactant subgroup. PI is the second most abundant anionic phospholipid, shares a common synthetic pathway with PG, and serves as a cross-functional phospholipid with PG (16). The decrease in PI could contribute to surfactant dysfunction in addition to the decrease in PG.

Although many mechanisms might contribute to the changes in phospholipid composition, one direct mechanism is hydrolysis by sPLA2s. Our group has previously demonstrated the presence of increased levels of sPLA2 in antigen-challenged models of asthma as well as allergic rhinitis (5, 32). In the current study, we demonstrated markedly increased sPLA2 hydrolysis of PG and PC in the BAL supernatant from asthmatics after antigen challenge. Although the increase in PG hydrolysis in the dysfunctional surfactant subgroup was greater than in the normal surfactant subgroup, the difference did not achieve statistical significance. Our group has demonstrated that the sPLA2 family has markedly different activities against the individual surfactant phospholipids (20). It is possible that multiple sPLA2s are active in the antigen-challenged BALF and that an sPLA2 with preference for PG is increased in the dysfunctional surfactant subgroup. The presence of an sPLA2 with a preference for PG would be more difficult to identify in the presence of hydrolysis from other sPLA2s. Our current data potentially support this possibility, since hydrolysis of PC is increased equally in both surfactant function subsets and could represent activity of an sPLA2 that is equally active against both phospholipids. The lack of significance in PG hydrolysis in the dysfunctional surfactant group may also indicate that other mechanisms unrelated to sPLA2 activity may contribute to differences in the phospholipids. For example, differences in synthesis, storage, or release of surfactant phospholipid, in particular PG, in response to an injury or stress such as antigen challenge could also play a role.

Generation of lysophospholipids is typically considered the major pathogenic mechanism for sPLA2-mediated abnormalities within phospholipids. In our analysis, we were unable to identify significant increases in either LPC or LPG. However, the absence of lysophospholipids is likely the result of their rapid reuptake by cells within the airway microenvironment, whereby the lysophospholipid is reacylated and used for new phospholipid biosynthesis (8). Despite the absence of lysophospholipids in the BALF after antigen challenge, the decreased PG observed in the dysfunctional surfactant group suggests sPLA2 may be an important contributing mechanism. In separate studies, we have demonstrated in vitro that sPLA2-mediated surfactant dysfunction is more dependent on the depletion of PG than on the generation of lysophospholipids for several phospholipases (19).

In summary, our study confirms the importance of surfactant abnormalities in asthma and provides new insights into the possible pathogenic mechanisms. Although our data confirmed the previously reported increase in protein and eosinophils after allergen challenge, our analysis suggests that these are not likely to be sufficient mechanisms to fully explain the functional variability seen within these subjects and that other surfactant-specific end points are important. Of these end points, an absolute reduction in PG and its subsequent contribution to the relative changes in PC/PG correlated most strongly with surfactant dysfunction. The decreased PG content along with the potentially causative increase in sPLA2 activity are important observations, as each could represent specific targets for therapeutic manipulation.

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Some of these results have been previously reported in the form of abstracts.

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DISCLOSURES

Since 1996, R. D. Hite has served on the advisory board for ongoing clinical trials in acute lung injury with an investigational synthetic surfactant, Surfaxin (Discovery Laboratories, Inc., Doylestown, PA). During that time, Dr. Hite received stocks as a compensation for his efforts related to that activity. He holds shares of Discovery Laboratories stock (DSCO) and has received direct payments for his consultancy with Discovery Laboratories, Inc.

REFERENCES


