Lysophospholipid generation and phosphatidylglycerol depletion in phospholipase A2-mediated surfactant dysfunction

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Hite, R. Duncan, Michael C. Seeds, Anca M. Safta, Randolph B. Jacinto, Julianna I. Gyves, David A. Bass, and B. Moseley Waite. Lysophospholipid generation and phosphatidylglycerol depletion in phospholipase A2-mediated surfactant dysfunction. Am J Physiol Lung Cell Mol Physiol 288: L618 –L624, 2005. First published October 29, 2004; doi:10.1152/ajplung.00274.2004.—Pulmonary surfactant’s complex mixture of phospholipids and proteins reduces the work of breathing by lowering alveolar surface tension during respiration. One mechanism of surfactant damage appears to be the hydrolysis of phospholipid by phospholipases activated in the inflamed lung. Humans have several candidate secretory phospholipase A2 (sPLA2) enzymes in lung cells and infiltrating leukocytes that could damage extracellular surfactant. We considered two mechanisms of surfactant disruption by five human sPLA2s, including generation of lysophospholipids and the depletion of specific phospholipids. All five sPLA2s studied ultimately caused surfactant dysfunction. Each enzyme exhibited a different pattern of hydrolysis of surfactant phospholipids. Phosphatidylcholine, the major phospholipid in surfactant and the greatest potential source for generation of lysophospholipids, was susceptible to hydrolysis by group IB, group V sPLA2s, but not group IIA or IID. Group IIA hydrolyzed both phosphatidylethanolamine and phosphatidylglycerol, whereas group IID was active against only phosphatidylglycerol. Thus, with groups IB and X, the generation of lysophospholipids corresponded with surfactant dysfunction. However, hydrolysis of and depletion of phosphatidylglycerol had a greater correlation with surfactant dysfunction for groups IIA and IID. Surfactant dysfunction caused by group V sPLA2 is less clear and may be the combined result of both mechanisms.

Lung injury; asthma; surface tension

THE PRINCIPAL FUNCTION of pulmonary surfactant is to reduce the work of breathing by lowering alveolar surface tension during respiration. This lowering of air-liquid interfacial tension is accomplished by the complex mixture of phospholipids (90%) and proteins (10%), which are produced and released by type II epithelial cells into the alveolus (27, 33). Changes in the ratio of the mixture can dramatically alter the surface tension in small airways and alveoli, compromising airway patency, gas exchange, and host defense. Consequently any injury to surfactant can have severe pathological consequences to lung function.

Phosphatidylcholine (PC) predominates surfactant phospholipid composition (~80% of total lipid), and the majority of the fatty acid side chains at the sn-1 and sn-2 positions are saturated or monounsaturated (39). Phosphatidylglycerol (PG) typically makes up only 10% of total surfactant phospholipid but plays a critical role in phospholipid-protein interactions that maintain the alveolar surfactant layer, especially through interactions with surfactant protein B (SP-B) (7, 31). Phosphatidylethanolamine (PE) is also present (~5%), whereas other phospholipids, free fatty acids, and neutral lipids make up the remaining surfactant components (39). The proteins include two hydrophilic surfactant proteins, SP-A and SP-D, which contribute to surface tension-lowering activity and play significant roles in innate immunity (29). Two hydrophobic proteins, SP-B and SP-C, predominantly contribute to surface tension-lowering activity through the organization of tubular myelin and the adsorption and redistribution of the surfactant film at the air-liquid interface (31).

Prior studies from our laboratories and others have supported the role of secretory phospholipase A2s (sPLA2s) in inflammation-mediated surfactant dysfunction (9, 20). sPLA2s are released by a variety of cell types and hydrolyze phospholipids at the 2-acyl ester position generating lysophospholipids and free fatty acids. At least 10 functional mammalian sPLA2 genes have been identified, and nine genes are expressed in humans (3, 8, 11, 17, 23). The sPLA2s are categorized by their small molecular size (13–16 kDa); an absolute requirement for calcium, which is involved in catalysis; and the presence of at least six highly conserved disulfide bonds that maintain the tertiary structure and serve as a basis for these enzymes’ nomenclature (36).

Lung tissue contains numerous cell types that could be sources of sPLA2 during asthma-induced inflammation. We have detected mRNA for groups IID, V, and X sPLA2s in human airway epithelial cells and group IID mRNA in human eosinophils (28, 34). Antibodies have confirmed group X protein in airway epithelium as well (18). Group V and group X mRNAs, but not group IID, were recently detected in purified neutrophils by RT-PCR and Western blotting (12).

In previous work, we and others have demonstrated that generation of lysophospholipids is an important potential mechanism of sPLA2-mediated surfactant injury (9, 20, 22), but it may not fully account for dysfunction caused by sPLA2s (24). Free fatty acids, which are generated in equimolar amounts to lysophospholipids as a result of sPLA2 hydrolysis, can impact surface activity, but the impact is more variable and typically far less in magnitude than seen for lysophospholipids (22). In most studies, hydrolysis...
of surfactant is assessed primarily by sPLA₂-mediated hydrolysis of PC, specifically dipalmitoylphosphatidylcholine (20, 22), which is the most abundant surfactant phospholipid (70–80%). However, only two human sPLA₂ (group V and group X) have shown significant ability to bind and hydrolyze PC relative to other phospholipid head groups (35). Therefore, hydrolysis of other surfactant phospholipids present may also contribute to phospholipase-mediated effects on surface tension-lowering activity.

In this study, we measured the effect of five different mammalian sPLA₂s (groups IB, IIA, IID, V, and X) on their capacity for hydrolysis of the three principal phospholipids (PC, PG, and PE) and their corresponding ability to generate surfactant dysfunction.

METHODS

Phospholipid labeling. 1-α-Dipalmitoyl-[2-palmitoyl-9–14C]-phosphatidylcholine (¹⁴C-DPPC) was purchased from New England Nuclear (Boston, MA). Radiolabeled 1-[¹⁴C]glycerol-PG and 1-[¹⁴C]glycerol-PE were isolated from ¹⁴C-glycerol-labeled Escherichia coli as described (1). Purification of the radiolabeled PG and PE was performed by lipid extraction (4), and separation via thin-layer chromatography (TLC) (15). Samples were stored as a chloroform solution after analysis of radioactivity and phosphorous content (2).

Surfactant labeling. We labeled samples of Survanta (Ross Laboratories, St. Louis, MO) daily by initially drying an aliquot of radiolabeled lipid under N₂ (100,000 dpm/mg of total phospholipid) in a glass mixing tube to which Survanta was added. Repetitive vortexing at 37°C over a minimum of 30 min was used to incorporate the radiolabeled lipid under N₂ (100,000 dpm/mg of total phospholipid) into the native surfactant phospholipids as described at the methods of Bartlett (2). After labeled phospholipids were mixed with surfactant, the concentration of the specific phospholipid (total of unlabeled and labeled) was not significantly increased (<10% increase of the expected concentration for the individual phospholipid).

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Surfactant function. We used a pulsating bubble surfactometer (Electronetics, Amherst, NY) that models the in vivo action of the alveolus. The bubble was cycled (20 cycles/min) within the liquid sample from minimal radius (0.40 mm) to maximal radius (0.55 mm) for 10 min at 37°C, and the pressure difference (ΔP) across the air-liquid interface was measured allowing calculation of surface tension (γ) according to the law of Laplace, ΔP = 2γ/r (13). The γmin represents the minimum surface tension of at least three consecutive minutes achieved during each 10-min run (20). Control experiments were performed with equal volumes of supernatant from mock-transfected cells for each cloning system, and no significant changes in γmin were seen (data not shown).

RESULTS

Surfactant hydrolysis and dysfunction. To carefully examine the mechanisms by which sPLA₂s lead to surfactant dysfunction, we measured the in vitro ability of five sPLA₂s to disrupt the surface tension function and hydrolyze the radiolabeled phospholipids in a bovine lung surfactant extract, Survanta. The results of those experiments are outlined by each respective enzyme in Fig. 1.

All five sPLA₂s tested caused significant increases in the γmin of surfactant. The dose-response curves revealed that some enzymes caused a greater increase in surface tension than others and that each enzyme had a unique profile for phospholipid hydrolysis. Group IB porcine pancreatic sPLA₂ was purchased (Sigma, St. Louis, MO). Recombinant human group IIA sPLA₂ and group V sPLA₂ were produced in COS-1 cells transfected with plasmids containing the sPLA₂ cDNAs cloned by RT-PCR from human lung tissue as described (34). Group IID and group X were amplified from human lung tissue by RT-PCR using 21–31 bases from the start codon (plus a Kozak sequence leader) and stop codons as forward and reverse primers. Primers for group IID were: forward 5'-GCCCGCATGGAACCTTGACTGTGGTGGGC-3' and reverse 5'-TGGCCTTCTGACCCGGG-3'. Primers for group X have been described (34). The recovered cDNAs were sequenced and cloned into insect expression vectors (TOPO-TA system of Invitrogen, Carlsbad, CA) and expressed in High Five insect cells (Invitrogen). sPLA₂s were partially purified by extracting the culture supernates overnight in 0.09 M H₂SO₄, dialyzing with 0.05 M Tris·HCl (pH 7.4), and partially fractionating the enzymes over a 30,000 molecular weight cut off (MWCO) Amicon filter (Fisher Scientific, Pittsburgh, PA). Enzyme activities were determined by hydrolysis of radiolabeled E. coli prepared by the method of Kramer and Pepinsky (26) and used as described (20). In all experiments, we defined 1 unit of enzyme activity as the amount required to yield 50% of maximum E. coli membrane hydrolysis (20). Mock-transfected COS cell media and mock-transfected High Five insect cell media were used as negative controls, were found to lack sPLA₂ activity, and did not hydrolyze surfactant (data not shown).

Phospholipase activity. Radiolabeled surfactant (1 mg total phospholipid/ml) was incubated with varied concentrations of either group IB, IIA, IID, V, or X sPLA₂ for 2 h at 37°C. After hydrolysis, the samples underwent lipid extraction, and the recovered phospholipids, lysophospholipids, and fatty acids were separated by two different TLC methods according to the radiolabel used. Radiolabeled free fatty acids hydrolyzed from ¹⁴C-DPPC were separated from intact ¹⁴C-DPPC with a mixture of hexane-ethyl ether-formic acid (90:60:6 vol/vol/vol). To separate phospholipids and lysophospholipids for the phospholipids tagged with radiolabeled glycerol (¹⁴CHPG and ¹⁴CPE), the method of Fine and Sprecher was used (15). The products were located on the TLC plates using a Bioscan System 200 (Bioscan, Washington, DC) or I₂ vapor, scraped, and analyzed by radioactivity in a scintillation counter (Beckman LS1801). The percentage hydrolysis represents the percentage of the total radioactivity, which was recovered in the lysophospholipid for PG and PE hydrolysis, and free fatty acid fractions for PC hydrolysis.

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All five sPLA₂s tested caused significant increases in the γmin of surfactant. The dose-response curves revealed that some enzymes caused a greater increase in surface tension than others and that each enzyme had a unique profile for phospholipid hydrolysis. Group IB caused surfactant dysfunction with γmin > 20 mN/m at enzyme concentrations ≥ 500 U/ml (Fig. 1A). At that concentration, group IB sPLA₂ preferentially hydrolyzed PE within the surfactant mixture (Fig. 1A). Approximately 10 times more group IB sPLA₂ was required to achieve equivalent hydrolysis of PC or PG. Hydrolysis of PC and PG were essentially equal and significant at these higher concentrations of enzyme.

Group IIA caused a similar level of surfactant dysfunction (γmin ≥ 25 mN/m) at ~2,000 U/ml (Fig. 1B). Unlike group IB, the group IIA sPLA₂ hydrolyzed only minor amounts of PC compared with PG and PE (Fig. 1B). PG and PE hydrolysis by group IIA was equal throughout the concentrations of enzyme examined. Hydrolysis of >5% PC could not be achieved despite enzyme concentrations 5–10 times greater than that required to achieve 40% hydrolysis of PG or PE.

Similar to group IIA, group IID caused significant surfactant dysfunction with little ability to hydrolyze PC (Fig. 1C). Furthermore, group IID was strikingly unique by its preferential hydrolysis of PG. At concentrations achieving very high γmin values (>30 mN/m), this phospholipase did not hydrolyze as much PE as group IIA (up to 5%) and very little PC (<1%).

Group V also caused minimum surface tension to increase (Fig. 1D). However, this was the only enzyme tested that
showed a nearly equal efficiency for the hydrolysis of all three phospholipid head groups.

The group X enzyme generated surfactant dysfunction (Fig. 1E) and was most active against PE with nearly equal activity against PC. However, the group X enzyme displayed poor activity with PG, which is unique compared with the other four enzymes.

Generation of lysophospholipids. We and others have suggested that the presence of lysophospholipids in surfactant films leads to dysfunction (9, 19, 20, 22). To evaluate the
generation of lysophospholipids, it is important to recognize that the absolute molar quantity of each phospholipid hydrolyzed is not the same as the percentage of each phospholipid label hydrolyzed as provided in Fig. 1. However, the percent hydrolysis by an sPLA$_2$ of each phospholipid and the absolute content of the individual phospholipid in the labeled surfactant preparation were used to calculate the lysophospholipid generated from hydrolysis. Analysis of the hydrolysis data on the basis of calculated lysophospholipid generated (combined hydrolysis of PC, PG, and PE) is presented as a percentage of the total phospholipids in Fig. 2. Results are calculated based on the model surfactant composition, which was 86% PC, 5% PG, and 5% PE. These estimates were derived from separate quantitative analyses of several Survanta samples by high-performance liquid chromatography and evaporative light scatter detection (data not shown). The predominance of PC vs. PG or PE in the surfactant (17:1 mol ratio) means that at an equal percentage of hydrolysis, PC generates a far greater mass of lysophospholipid than PG or PE.

As shown in Fig. 2, the changes in surface tension generated by groups IB, V, and X increased concurrently with generation of total lysophospholipid. The slopes of the surfactant dysfunction curves caused by these three enzymes are variable, but it appears that a minimum lysophospholipid content of 8–10% was required to achieve a significant increase in $\gamma_{\text{min}}$. This finding is consistent with the previous models used to examine this relationship (14, 19). In contrast, nearly maximal dysfunction was caused by group IIA and IID sPLA$_2$s with very little generation of total lysophospholipids. Although the group IIA has only limited ability to hydrolyze PC, the calculated proportion of all three lysophospholipids [lysophosphatidylcholine (LPC), lysophosphatidylglycerol (LPG), and lysophosphatidylethanolamine (LPE)] is equal, due to the abundance of the PC pool. Group IID sPLA$_2$ predominately generated LPG with minor contributions of LPE and LPC and thus differed substantially from all other sPLA$_2$s studied.

**Depletion of specific phospholipids.** The data on generation of lysophospholipids do not provide a comprehensive explanation of surfactant dysfunction across the five sPLA$_2$s studied. In particular, this explanation seems least sufficient for the dysfunction demonstrated with both groups IID and IIA. Depletion of specific phospholipids, in particular PG, may be important for these enzymes where very limited hydrolysis of PC did not create a large amount of lysophospholipid. Accordingly, we analyzed loss of surfactant function in relation to the loss of each of the three phospholipids (Fig. 3). The group IID data provided the most striking observation, with significant surfactant dysfunction that could be attributed only to hydrolysis of PG. From the PG depletion curve in Fig. 3A, it appears that surfactant dysfunction arises at a point when PG drops below a level of $\leq$80% in this model. Similarly, the surfactant dysfunction caused by group IIA and group V sPLA$_2$s was observed to change dramatically at this same point on the PG

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**Fig. 2.** Correlation of surface tension with lysophospholipid generation. Using the hydrolysis data outlined in Fig. 1, we determined total lysophospholipid formation by calculation of lysophospholipids from the composition of individual phospholipids within the surfactant preparation. Results are means plotted against surface tension.

**Fig. 3.** Correlation of surface tension with depletion of specific surfactant phospholipid pools. Data from Figs. 1 and 2 were used to correlate the relationship between surface tension and the depletion of PG mass (A), PE mass (B), or PC mass (C) in mol percent.
depletion curve, although surface tension increased less steeply for these two enzymes. Thus PG loss may be one significant mechanism generating surfactant dysfunction for groups IIA, IID, and V. Depletion of PE (Fig. 3B) was also associated with increased surface tension induced by most sPLA2s, except for group IID, but the curve lacked the distinct inflection point seen with PG depletion. Finally, loss of PC was not a factor for either group IIA or group IID (Fig. 3C). However, groups IB, V, and X generated significant dysfunction with metabolism of the PC component, consistent with significant generation of LPC due to the high PC composition of surfactant.

**DISCUSSION**

Surfactant dysfunction contributes to the pathophysiology of inflammatory lung diseases including acute lung injury and asthma (20, 22, 24). Likewise, in vitro studies have demonstrated increased sPLA2 activity in the bronchoalveolar lavage (BAL) fluid of inflammatory airway disease and the ability of sPLA2 to hydrolyze surfactant and induce surfactant injury (16, 20, 22). Although the available in vitro studies support the potential role of sPLA2 in causing in vivo surfactant dysfunction, they have not defined a clear mechanism for sPLA2-induced surfactant dysfunction in vivo.

In this study we used a commercial surfactant model to examine the impact of multiple sPLA2-mediated mechanisms on the surface tension-lowering activity of surfactant. To test the spectrum of these mechanisms and their relevance to inflammatory lung diseases in vivo, we examined five different sPLA2 enzymes, four of which have been localized to lung tissue or to invasive leukocytes frequently found in the inflamed lung. Our data demonstrate that all five sPLA2s are capable of hydrolyzing surfactant-associated phospholipids, but the profile of each sPLA2 for hydrolysis of the specific phospholipids within surfactant is unique. In addition, the ability of sPLA2 to cause surfactant dysfunction may be dependent on the generation of lysophospholipids and/or the depletion of PG.

Our results suggest that sPLA2s that readily hydrolyze PC, groups IB and X, generate significant quantities of LPC and that the resulting surfactant dysfunction caused by those enzymes correlates with the accumulation of lysophospholipid (Fig. 2). This mechanism would apply to group X especially, which does not cause significant hydrolysis of PG. In contrast, the dysfunction generated by groups IIA and IID sPLA2 has little dependence on the hydrolysis of PC or generation of lysophospholipids. The inflection in the curve of surfactant dysfunction at the point of ≥20% depletion of PG for groups IIA and IID suggests that these changes in PG may explain the impact of those enzymes on surfactant surface tension. This relationship is most notable for group IID, where PG hydrolysis is the only significant phospholipid hydrolysis caused by the enzyme at the concentrations required to cause surfactant dysfunction. Although all sPLA2s will hydrolyze pure PG vesicles, the specific activity of each enzyme for a single substrate varies widely (35). By use of pure phospholipid vesicle models, the relative preference for PG over PC by group IID, and the opposite preference for PC over PG by group X has been observed (35).

The group V enzyme in our studies appears to be the most complex. The surfactant dysfunction caused by this enzyme is not clearly explained by the accumulation of lysophospholipids or the depletion of PG or any of the other individual phospholipids studied. We speculate that its ability to hydrolyze all phospholipids equally might lead to multiple mechanisms including generation of lysophospholipids and PG depletion contributing to the resulting enzyme-associated surfactant dysfunction.

Multiple studies have demonstrated the ability of lysophospholipids to disrupt the surface activity of phospholipid mixtures (9, 19, 20, 22). This mechanism is often considered the most probable explanation for sPLA2-mediated surfactant dysfunction in vivo. We and others have demonstrated that a lysophospholipid content of at least 10–15% of total phospholipids content is required to cause dysfunction of surfactant in vitro (20, 22). However, BAL analyses of inflammatory lung diseases, in which increased sPLA2 activity and surfactant dysfunction can be demonstrated, have typically not demonstrated sufficient levels of lysophospholipid to explain surfactant dysfunction, including studies we have performed in asthma and acute lung injury. This discrepancy in enzyme activity and evidence of enzymatic product, lysophospholipid, is most likely explained by the rapid cellular reuptake of lysophospholipids through most cellular membranes. The lysophospholipids can be rapidly reacylated into phospholipids or be further metabolized into lipid bodies which often appear in leukocytes in the inflamed lung (10, 38, 40).

The depletion of phospholipids, in total or as individual phospholipids, is also an important consequence of sPLA2 hydrolysis and may also serve as important mechanisms for surfactant dysfunction. The correlation of surfactant dysfunction with hydrolysis of PG for groups IIA and IID sPLA2 may reflect the critical interaction between PG, the most abundant anionic phospholipid, and SP-B in maintaining optimal function of the surfactant film at the air liquid interface (31). Furthermore, the content of SP-B and the other surfactant proteins may play important roles in regulating specific sPLA2 activities (6). The potential role of surfactant proteins can be addressed utilizing surfactant preparations with more complete surfactant protein contents than the relative protein poor commercial preparation, Sur-Fanta, used in these experiments.

Elevated PLA2 activity has been found in the BAL of asthmatics (5, 9), in bronchial pneumonia (21), in acute respiratory distress syndrome (ARDS) (37), and in several animal models of acute lung injury (25, 30). However, the specific sPLA2s have not been identified. During inflammation of the lung, several cells could secrete phospholipases into the alveolar lining fluid. Inflammation characterized by neutrophil infiltration, including burn injury, pneumonia, and ARDS, could result in the release of group V and X (12). In conditions characterized by eosinophil infiltration, including asthma, group IID sPLA2 expression is more likely and might therefore lead to surfactant dysfunction via PG depletion (28). Airway epithelial cells contain mRNA for groups IID, V, and X (34) and could release sPLA2 during injury or inflammation, resulting in surfactant hydrolysis and dysfunction. Levels and activity of sPLA2 can also be increased in the serum in conditions such as sepsis (group IIA) and pancreatitis (group IB) and may play a role when these conditions lead to acute lung injury (32). Thus unique profiles of sPLA2 may contribute to surfactant dysfunction.
through differing mechanisms during specific forms of inflammatory lung injury or disease.

In summary, sPLA2-mediated hydrolysis of surfactant phospholipids is a complex interaction between the individual enzyme and the multiple potential phospholipid substrates. There are several potential mechanisms that contribute to the impact of hydrolysis on surfactant dysfunction including at least the generation of lysophospholipids and depletion of PG. The depletion of PG may be a more important contributor to sPLA2-mediated surfactant dysfunction in vivo given the capacity of cells to respond to the presence of extracellular lysophospholipids.

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GRANTS

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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


