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Secretory phospholipase A_2 mediated depletion of phosphatidylglycerol in early Acute Respiratory Distress Syndrome

Michael C. Seeds, PhD[1,2], **Bonnie L. Grier, BA**[2], **Bruce N. Suckling, MD**[2], **Anca M. Safta, MD**[2], **David L. Long, BA**[1], **B. Moseley Waite, PhD**[3], **Peter E. Morris, MD**[2], and **R. Duncan Hite, MD**[2,1]

[1]Department of Internal Medicine/ Section on Molecular Medicine, Wake Forest University School of Medicine, Winston-Salem, NC 27157

[2]Section on Pulmonary, Critical Care, Allergy and Immunologic Diseases, Wake Forest University School of Medicine, Winston-Salem, NC 27157

[3]Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract

Background—Secretory phospholipases A_2 ($sPLA_2$) hydrolyze phospholipids in cell membranes and extracellular structures such as pulmonary surfactant. This study tests the hypothesis that $sPLA_2$ are elevated in human lungs during acute respiratory distress syndrome (ARDS) and that $sPLA_2$ levels are associated with surfactant injury by hydrolysis of surfactant phospholipids.

Methods—Bronchoalveolar lavage (BAL) fluid was obtained from 18 patients with early ARDS (<72 hours) and compared to samples from 10 healthy volunteers. Secreted phospholipase A_2 were measured (enzyme activity and enzyme immunoassay) in conjunction with ARDS subjects' surfactant abnormalities including surfactant phospholipid composition, large and small aggregates distribution, and surface tension function.

Results—BAL $sPLA_2$ enzyme activity was markedly elevated in ARDS samples relative to healthy subjects when measured by *ex vivo* hydrolysis of both phosphatidylglycerol (PG) and phosphatidylcholine (PC). Enzyme immunoassay identified increased PLA2G2A protein in the ARDS BAL fluid, which was strongly correlated with the $sPLA_2$ enzyme activity against PG. Of particular interest, we demonstrated an average depletion of 69% of the PG in the ARDS sample large aggregates relative to the normal controls. Furthermore, the $sPLA_2$ enzyme activity against PG and PC *ex vivo* correlated with the BAL recovery of *in vivo* PG and PC, respectively, and also correlated with the altered distribution of the large and small surfactant aggregates.

Conclusions—These results support the hypothesis that $sPLA_2$-mediated hydrolysis of surfactant phospholipid, especially PG by PLA2G2A, contributes to surfactant injury during early ARDS.

Address reprint requests to the Corresponding Author: R. Duncan Hite, M.D., Professor of Internal Medicine, Section on Pulmonary, Critical Care, Allergy and Immunologic Diseases, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC, 27157-1054, Phone: (336) 716-4649, Fax: (336) 716-7277, dhite@wfubmc.edu.

Keywords

Acute Lung Injury; Adult Respiratory Distress Syndrome; Secretory Phospholipase A2; Pulmonary Surfactant; Phosphatidylglycerol

Introduction

The alveolar inflammation in acute lung injury (ALI) and acute respiratory distress syndrome leads to changes in the biophysical and biochemical properties of pulmonary surfactant causing injury that contributes to the disease severity [1,2]. One contributing mechanism by which surfactant injury arises with inflammation may involve increased levels of secretory phospholipases A_2 in the lung [3,4,5].

Phospholipase A_2 (PLA_2) enzymes hydrolyze the *sn*-2 fatty acid from phospholipids yielding lysophospholipid and free fatty acid products. Mammalian PLA_2 can be generally characterized as cytosolic or secreted enzymes. The secretory phospholipases ($sPLA_2$) are especially suited for extracellular function as they are small in size (~ 14 kDa), are extensively disulfide bonded and stable in extracellular environments, and are enzymatically active in millimolar calcium levels [6]. Thus, $sPLA_2$ can hydrolyze cellular outer membrane phospholipids (PL) as well as PL in extracellular structures such as pulmonary surfactant. $sPLA_2$ have been reported to be elevated in a variety of inflammatory or infectious diseases [7,8].

Multiple $sPLA_2$ have been identified in human lung [9,10,11,12] and several studies have reported elevated levels of $sPLA_2$ activity in serum or BAL fluid from patients with ALI/ARDS, which may relate to lung injury [3,13,14]. Surfactant films appear to be biological targets of $sPLA_2$ *in vitro* [15,16,4]. However, while several animal studies indicate $sPLA_2$ are linked to changes in surfactant composition and function [5,17], data from human samples documenting the identification of specific $sPLA_2$ proteins or their *in vivo* role in lung injury or surfactant injury are limited [3,18,19].

Injury of surfactant in both animal and human studies of ALI and ARDS results in impaired surfactant function, potential alveolar collapse and impaired gas exchange and reduced lung compliance [13]. Pulmonary surfactant is a complex mixture of phospholipids (80-90% by weight), neutral lipids (5-10%), and proteins (5-10%) that primarily serves to lower surface activity, maintaining both alveolar and airway patency [2,20]. Surfactant phospholipids consist predominantly (80%) of phosphatidylcholine (PC). Although phosphatidylglycerol (PG) comprises only about 10% of human pulmonary surfactant PL, this anionic phospholipid plays a critical role in maintaining surfactant function, in part through its interactions with the essential hydrophobic surfactant protein B (SP-B) [21]. Among the multiple changes in surfactant composition in ALI and ARDS, several studies have demonstrated a significant, disproportionate loss of PG [22,23]. A clear mechanism for this PG depletion has not been reported.

This study was designed to test the hypothesis that increased levels of $sPLA_2$ in the lung results in surfactant injury during ARDS, in part by changing surfactant composition through hydrolysis of PL. BAL samples were obtained early (≤ 72 hours) in the course of ALI/ARDS and both $sPLA_2$ and surfactant properties were measured to identify mechanisms of injury.

Methods

Patient Selection

BAL samples from ARDS patients were obtained within 72 hours of onset of ARDS (n=18), and all subjects met the American-European Consensus Conference diagnostic criteria for ALI/ARDS [1]. Subjects were consented by surrogates according to protocols approved and licensed by the Institutional Review Board. BAL samples from consenting healthy volunteer control subjects (n=10) were obtained in the Wake Forest University Health Sciences General Clinical Research Center under protocols approved and licensed by the Institutional Review Board.

BAL processing

BAL was obtained using 3 × 50 ml aliquots of normal saline in either the right medial lobe or lingula. The total BAL was pooled and centrifuged at 200 × g for 15 minutes to remove cells. The supernatant was centrifuged at 40,000 × g for 1 hour and the resulting surfactant pellet large aggregates (LA) washed twice with normal saline, re-suspended in a known volume of saline, aliquoted and stored at -70°C. The supernatant was aliquoted and stored at -70 °C until assayed for $sPLA_2$. Small aggregates (SA) were defined as the surfactant PL remaining in the 40,000 × g supernatant [18]. The phospholipid content of LA and SA was measured by the method of Rouser [24]. Total surfactant recovered was calculated as the sum of LA and SA phospholipids. Adequate material was obtained to complete analysis of 16-18 ARDS samples for each endpoint assay. Total protein of each surfactant fractions was measured using a BCA assay (Thermo Fisher Scientific, Rockford, IL).

Phospholipase activity

$sPLA_2$ activity in BAL supernatants (475 μl) were measured by hydrolysis of a model surfactant (Survanta®, Ross Laboratories; St. Louis, MO) at 1mg/ml containing trace-labels of either [^{3}H]-dipalmitoyl-PC (100 μCi/μmol, ~100,000 dpm/sample, American Radiolabeling Company, St. Louis, MO), or [^{3}H]-PG (10 μCi/μmol, ~30,000 dpm/sample) for 4 hours as described [16]. Hydrolysis was calculated from the radioactivity in the free fatty acid ([^{3}H]-PC) or lysophospholipid ([^{3}H]-PG) fractions and is graphed in units of total pmol of phospholipid hydrolyzed per sample.

Characterization of $sPLA_2$ protein

$sPLA_2$ protein from BAL fluid was measured with a human Type IIA $sPLA_2$ Enzyme Immunoassay (EIA) Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. This EIA is not reactive with either PLA2G1B, PLA2GIV or PLA2GV.

Surfactant Phospholipid Composition

Phospholipid composition of the LA fraction was determined using HPLC with electronic light scatter detection (ELSD, SEDERE, Alfortville, France) using phosphatidylbutanol (PB) as an internal standard as described [16]. Data for individual phospholipids are presented as percentage of total LA phospholipid, or as μg of PL/ml of BAL fluid.

Surfactant Function

A pulsating bubble surfactometer (General Transco, Seminole, FL) was used to measure surface tension lowering activity from the LA fraction at a concentration of 1.0 mg phospholipid/ml as described previously [15]. The results are reported as γ_{min}, the minimum stable surface tension achieved over 10 minutes.

Statistics

Comparison of medians between ARDS samples and controls were made with Mann Whitney rank sum tests due to unequal variance of the populations. Table data are presented as median with interquartile range (75% - 25%). Correlations for strength of association between ARDS sample surfactant parameters were calculated using Pearson or Spearman methods as appropriate for the distribution of the surfactant parameter measured (SigmaStat, Systat Software, Inc, Point Richmond, CA).

Results

Secretory phospholipase A_2 activity and PLA2G2A protein increased during early ARDS

The $sPLA_2$ enzymatic activities in each ARDS BAL supernatant were measured *ex vivo* to ascertain if the enzyme was present as a potential mechanism for surfactant PL degradation. Table 1 demonstrates that the ARDS samples $sPLA_2$ enzymatic activities were significantly increased relative to the healthy controls. This was true for hydrolysis of both PG ($p < 0.001$) and PC ($p < 0.05$) substrates.

We and others have reported that the Group 2A subtype (PLA2G2A) of the $sPLA_2$ family demonstrates a high affinity for PG, and relatively poor affinity for PC [16,25]. Given the elevated $sPLA_2$ enzymatic activity in these BAL samples, we measured PLA2G2A protein by EIA to identify the enzyme. As shown in Table 1, the PLA2G2A levels in the BAL supernatant of the healthy volunteers were low on average, and two of the ten samples were below the sensitivity of the EIA (15 pg/ml). In contrast, PLA2G2A levels in the ARDS patients were elevated on average over 30 fold relative to the healthy controls ($p < 0.001$). When compared to the enzymatic activity of the BAL, a strong correlation was seen between the *ex vivo* PG hydrolysis and PLA2G2A in the ARDS samples (Figure 1A, $R = 0.82$, $p < 0.0001$). Of note, a significant though weaker correlation was also seen between PC hydrolysis and PLA2G2A protein (Figure 1B, $R = 0.51$, $p < 0.05$).

Surfactant abnormalities in early ARDS

Composition of surfactant phospholipids was measured by HPLC analysis of the LA fractions (Figure 2) to determine potential changes in ARDS surfactants. As anticipated, PC was most abundant as a percent of total PL and did not differ statistically between the ARDS patients' samples (81 ± 11.5% of LA PL, median ± standard deviation) and healthy volunteers (81.4 ± 3.9% of LA PL). However, a particularly striking loss of PG was seen in the ARDS samples, which were depleted to only 31% of PG levels measured in the healthy donor surfactant LA fraction (4 ± 3.5% of LA PL in ARDS vs 11.7 ± 3.7% in the controls, $p < 0.001$). This PG deficiency was accompanied by a significant increase in phosphatidylinositol (from 2.3 ± 1.1% of LA PL in controls to 4.6 ± 2.2% in ARDS, $p < 0.001$). No significant change was seen in other phospholipids, including phosphatidylethanolamine (PE). Our methods included attempts to measure both lysophosphatidylcholine (LPC) and lysophosphatidylglycerol (LPG), but significant levels were not seen in either the normal or ARDS samples. Sphingomyelin, predominantly a cellular sphingolipid, was elevated in the ARDS LA samples (from 0 ± 0.8 to 3.9 ± 9.5% of LA PL, $p < 0.001$).

Consistent with previous reports, multiple other surfactant-associated abnormalities were identified in the BAL material isolated from these early ARDS patients, as shown in Table 2. Surface tension lowering activity (γ_{min}) of the surfactant LA fractions from the ARDS patients exhibited significantly greater γ_{min} compared to surfactant from healthy volunteers ($p < 0.001$). Furthermore, the distribution of surfactant phospholipid between LA and SA (LA/SA ratio) was significantly decreased in the ARDS subjects relative to the healthy

subjects ($p < 0.001$). Of note in our study, the decreased LA/SA ratio in the ARDS samples trended to decrease with decreased LA recovery within this cohort ($R = 0.71$, $p < 0.01$, $n = 17$, not shown) even though average LA total PL did not change significantly compared to controls. The decreased LA/SA ratio also correlated inversely with increased SA ($R = - 0.6$, $p = 0.01$) in these samples, and SA increased in parallel with total phospholipid recovery (LA + SA; $R = 0.85$, $p < 0.001$, not shown).

Increased capillary permeability and increased serum protein influx are important components in surfactant injury and dysfunction during ARDS [26,27]. The protein concentrations in both the SA and LA fractions were significantly elevated in our ARDS samples (Table 2) despite washing the LA pellets.

To identify factors affecting surfactant function, correlation analyses were performed between the significantly greater γ_{min} values from the ARDS LA fractions and multiple BAL measurements, including aggregate distributions, phospholipid compositions, and protein. There was a significant inverse correlation between LA phospholipid and γ_{min} ($R = - 0.72$, $p < 0.001$, not shown). Consistent with the depletion of total LA, recovery of the most abundant individual PLs within the LA, PC and PG, also correlated inversely with γ_{min} ($R = -0.64$ and $R = -0.55$, respectively; $p < 0.05$, not shown). No other significant correlations between changes in PLs and γ_{min} were identified. Despite the increase in SA and LA total protein, the correlation between those protein levels and increased γ_{min} of the ARDS LA fractions was not significant.

Association of $sPLA_2$ with surfactant injury

The $sPLA_2$ enzymatic activities from ARDS BAL supernatants were compared to the recovery of surfactant aggregates. As PG hydrolysis activity increased, the LA/SA ratio of the ARDS samples decreased ($R = -0.63$, $p < 0.01$, Figure 3), but this was not true for PC hydrolysis activity ($R = -0.36$, $p = 0.16$, not shown). However, recovery of total LA or SA did not correlate with $sPLA_2$ enzymatic activity against PG or PC.

Furthermore, the $sPLA_2$ enzymatic activities from ARDS BAL supernatants were compared to the recovery of individual surfactant PL. $sPLA_2$ activities, measured as PG hydrolysis, were inversely correlated with absolute recovery of PG in the LA fractions ($R = -0.561$, $p < 0.05$, not shown). The same trend was seen between PC hydrolysis activity and absolute recovery of PC in the LA fractions, which approached statistical significance ($R = -0.46$, $p = 0.07$, not shown). Further analyses demonstrated no significant correlation was seen between hydrolysis of PC and recovery of PG nor between hydrolysis of PG and recovery of PC. There was also no correlation between PG hydrolysis and recovery of PI, though PI was generally increased in the ARDS BALs (Figure 2).

Discussion

This study was designed to test the hypothesis that increased $sPLA_2$ activity in ARDS lungs is linked to surfactant abnormalities, and our results identified a strong correlation between the BAL $sPLA_2$ activity and PLA2G2A protein, with a stronger correlation for hydrolysis of PG than PC. Our results also identified marked reduction in PG in the LA fractions with an inverse correlation between BAL sPLA2 PG hydrolysis and PG recovery. The reductions in PG have been previously reported as have other alterations in surfactant composition demonstrated in our studies (increased PI and sphingomyelin), decreased LA/SA ratio, and increased γmin, which indicates our sample set is representative of ARDS/ALI patients included in previous reports [28,23,3].

The strength of our observations reflects the measure of $sPLA_2$ and several surfactant endpoints in the same human BAL samples. Our data are consistent with animal models that indicate $sPLA_2$ can change surfactant PL composition and that inflammatory stimuli are linked to increased $sPLA_2$ levels, in particular to PLA2G2A, which is induced by cytokines such as TNFα and IL-6 [29,30,31]. Our study focused on the early (<72 hour) phase of ARDS during which those cytokines are known to be up-regulated [32,33]. The results of our EIA analysis indicate the presence of PLA2G2A in ARDS BAL and are consistent with the findings of Nakos et al., who measured PLA2G2A by western blots of plasma and BALs from mechanically ventilated ICU patients [12]. The stronger correlation with PG hydrolysis in our samples is consistent with the known enzymatic characteristics of PLA2G2A [25]. However, the significant correlation with PC hydrolysis rates suggests that other $sPLA_2$ may be present. Greater affinity for PC hydrolysis is a characteristic of several $sPLA_2$, including PLA2GV and PLA2GX [25,16]. PLA2GX protein has also been observed in airway epithelial cells in asthmatics [34], and knockout mice lacking PLA2GV have diminished ALI in response to intratracheal LPS administration [35].

The source of the $sPLA_2$ in these early ARDS BAL samples is unknown. PLA2G2A mRNA or protein have been reported in airway epithelial cells [9,34] and in serum during systemic infection or inflammation [36,12]. In rodent models, macrophages produce PLA2G2A in response to cytokine-stimulation and infection-induced models of ALI/ARDS [37,38]. Data for PLA2G2A from human leukocytes is sparse, though blood macrophages are reported to produce PLA2G2A in atherosclerosis [39]. We have observed immunohistochemical staining for PLA2G2A in the BAL macrophages and neutrophils from ARDS patients (Seeds and Hite, unpublished). However, unstimulated human peripheral blood neutrophils do not contain mRNA for PLA2G2A, but do contain PLA2GV and PLA2GX [34,40,35]. Elevation of BAL PLA2G2A levels may also represent plasma leaks of increased systemic $sPLA_2$.

The depletion of PG seen in our results may be an important contributing mechanism to surfactant injury in ALI/ARDS. PG comprises approximately 10% of phospholipid in human pulmonary surfactant, but plays an important role by enhancing phospholipid adsorption to the air-liquid interface and re-spreading after surfactant films are compressed [41]. Recent studies have also suggested that PG plays a significant role in suppressing pulmonary inflammation [38], specifically during viral infection [42].

Increased PI, a second anionic surfactant phospholipid, was also measured in our samples, and has been observed in previous analyses of ALI/ARDS BAL fluid [43]. PG and PI share a common initial synthetic pathway, thus, surfactant anionic phospholipid synthesis may be intact in our samples, though PI levels could reflect either altered synthesis or decreased consumption of PI [43]. Interestingly, PG and PI vary in proportion in several mammalian species [44], but it is not known that PI is equally functional to PG in humans.

The generation of lysophospholipids and free fatty acids are the hallmark of PLA_2 hydrolytic activity, *in vitro*. In our previous studies, *in vitro* addition of lysophospholipids caused significant surfactant dysfunction, however addition of saturated and monounsaturated free fatty acids did not [45]. Our HPLC analysis did not demonstrate lysophospholipids in our ARDS BAL samples, but this is likely consistent with the uptake and reacylation of these highly toxic lysophospholipids by intact cells *in vivo* [46]. Free fatty acids were increased in the HPLC analysis of the ARDS BALs relative to controls (not shown), but characterization of individual fatty acid species was not performed.

During ALI, the rate of surfactant aggregate conversion increases and may contribute to the pathology of the injury [47]. Our results demonstrate a correlation between PG hydrolysis and a shift in the LA/SA ratio (Figure 3), and a separate significant correlation between LA

recovery and surfactant function. Although intriguing, these results alone do not represent direct evidence to support LA/SA shift as a mechanism for $sPLA_2$-mediated surfactant dysfunction. When surface tension was measured in the ARDS samples, 14 of the 18 samples had surface tensions of greater than 20 mN/m, whereas the healthy control surfactant values were less than 5 mN/m on average. This creates a dichotomous endpoint such that not even protein levels, a known factor in surfactant injury, correlated with surfactant function, γmin (not shown). Consequently, while increased $sPLA_2$ levels in ARDS BALs appear to be linked to changes in surfactant composition consistent with injury, we cannot yet say that it is sufficient to cause dysfunction in a complex, multifactorial inflammatory disease such as ARDS.

Multiple trials of surfactant replacement therapy (SRT) in adults with ARDS have not demonstrated improved clinical outcomes [48,49] despite its success in the treatment of neonatal RDS [50]. A significant hurdle may be that the presence of severe inflammation of ARDS during SRT leads to degeneration of the exogenously administered surfactant [28]. In this context, the kinetics of $sPLA_2$ synthesis and release relative to surfactant injury will be important to define.

In conclusion, among the numerous inflammatory processes that lead to lung and surfactant injury in early ARDS, increased levels of PLA2G2A protein and enzymatic activity is an important contributor that provides a mechanism for depletion of pulmonary surfactant PG and possibly a shift from LA to SA. Our results highlight the need to better characterize the types of $sPLA_2$ present, the cells and cytokines responsible for their up-regulation and the functional impact of the resulting surfactant PL alterations.

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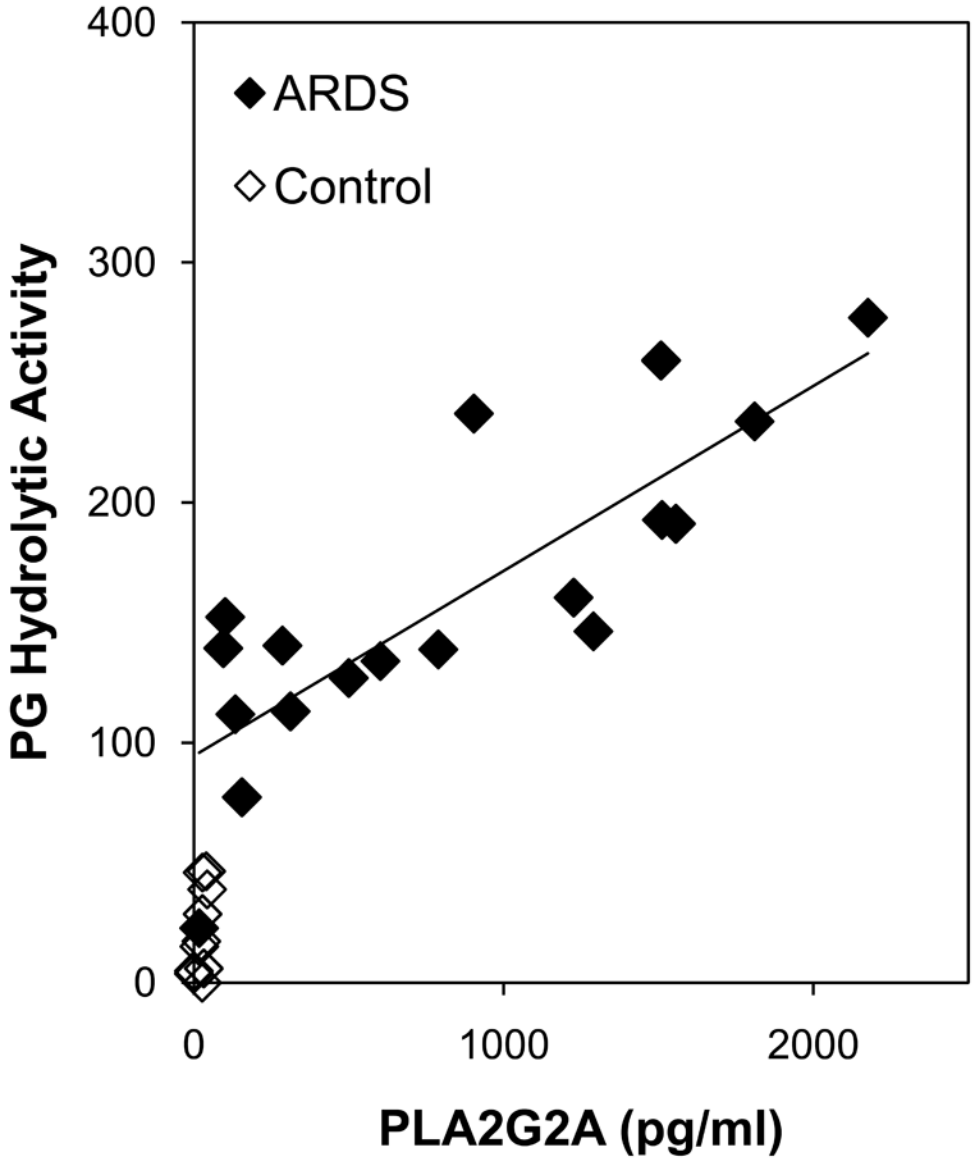

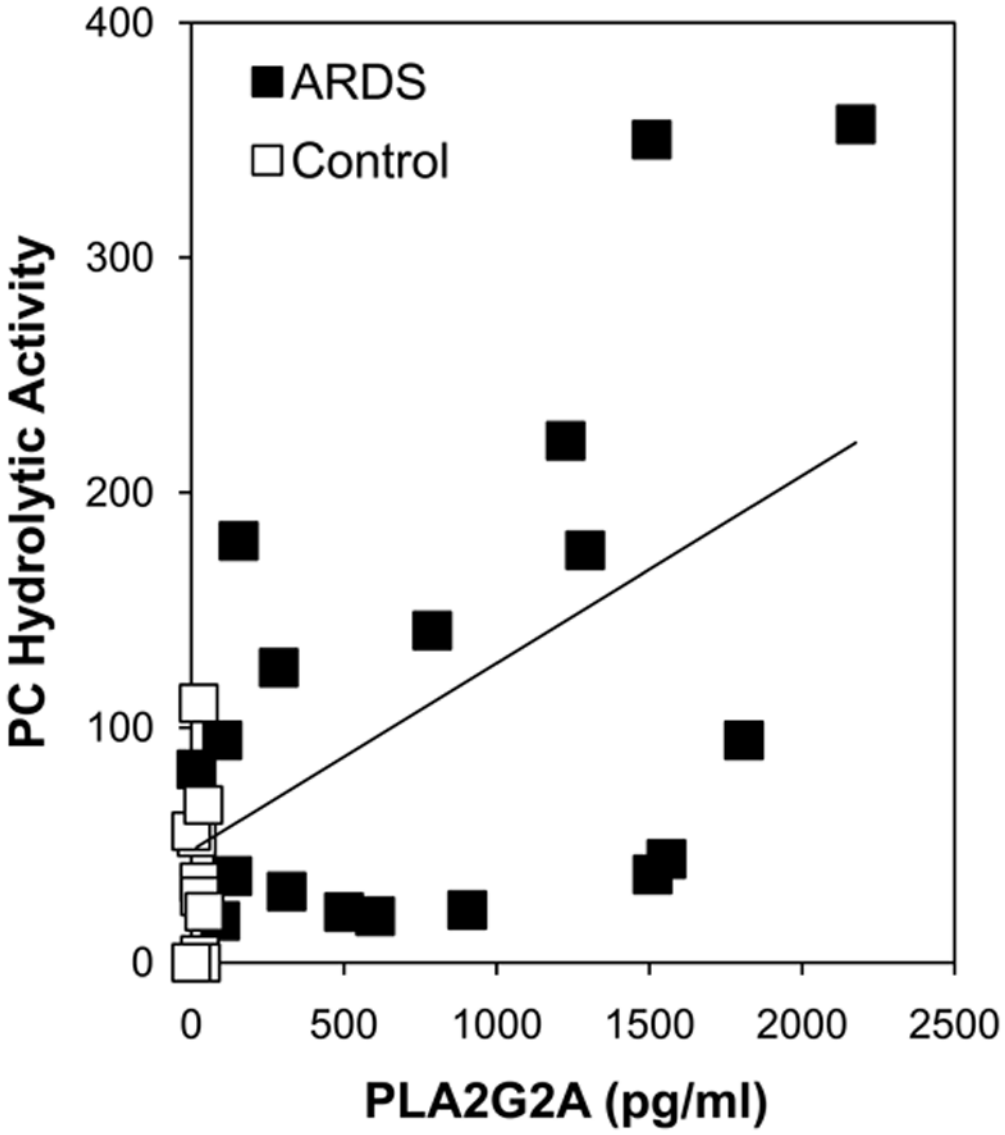

Figure 1. sPLA$_2$ enzymatic activity vs PLA2G2A protein content from BAL

PLA2G2A protein was measured by EIA (x-axis) and compared in ARDS BAL samples to healthy normal controls. Data are shown relative to the amount of sPLA$_2$ *in vitro* hydrolysis of phosphatidylglycerol (PG) (**A**) or phosphotidylcholine (PC) (**B**) in a surfactant substrate (y-axis). PLA2G2A correlated with PG hydrolytic activity ($p < 0.0001$) and with PC hydrolytic activity ($p < 0.05$) from the ARDS BAL samples (Pearson correlations, n=18).

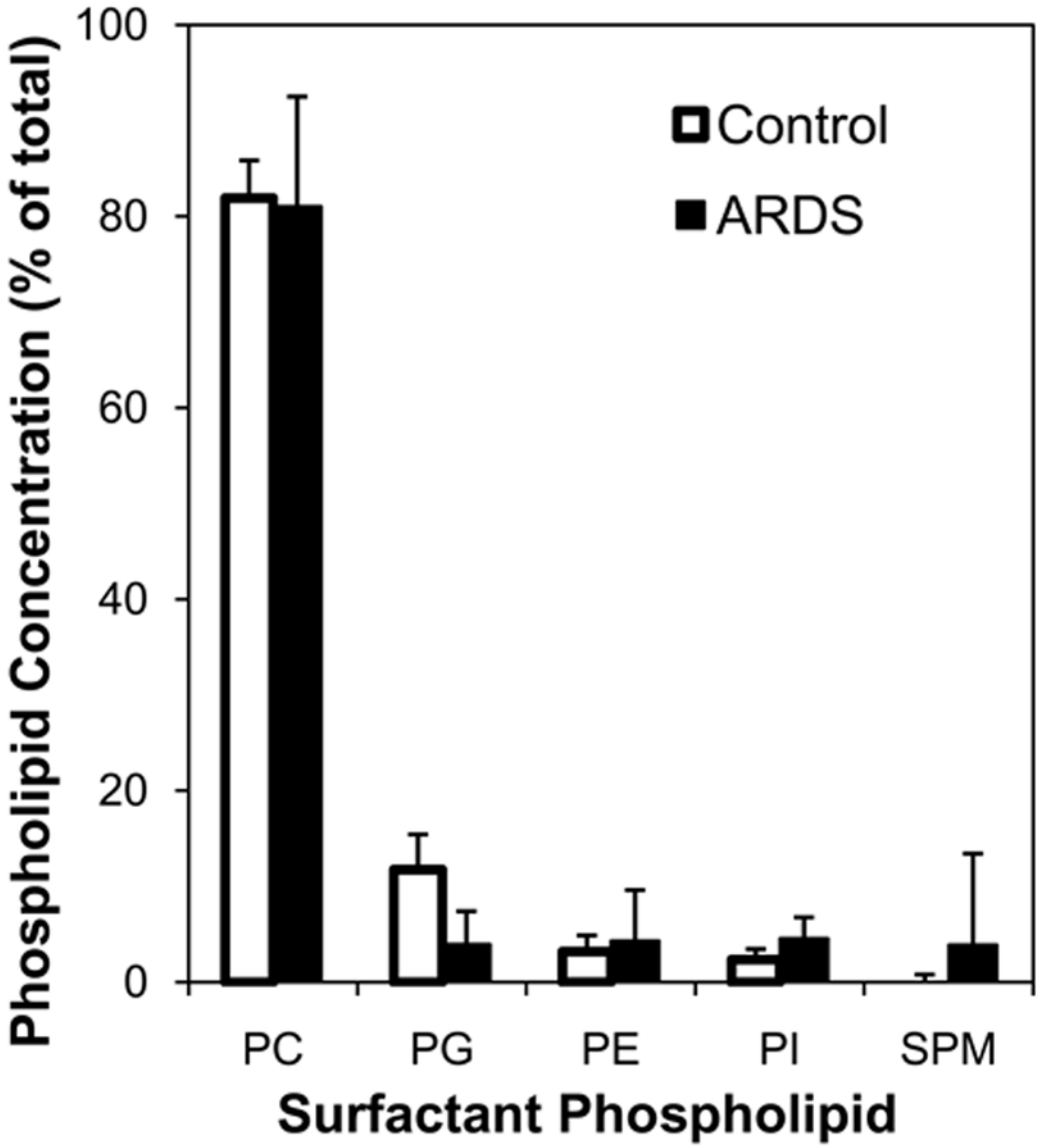

Figure 2. Phospholipid composition of LA fractions shows depletion of PG in ARDS

The LA fractions from early ARDS BALs were analyzed for phospholipid composition by HPLC. Depletion of PG was noted in the ARDS samples relative to healthy control BALs, whereas PI and sphingomyelin increased (median ± standard deviation, * $p < 0.001$, Mann Whitney rank sum, n=16).

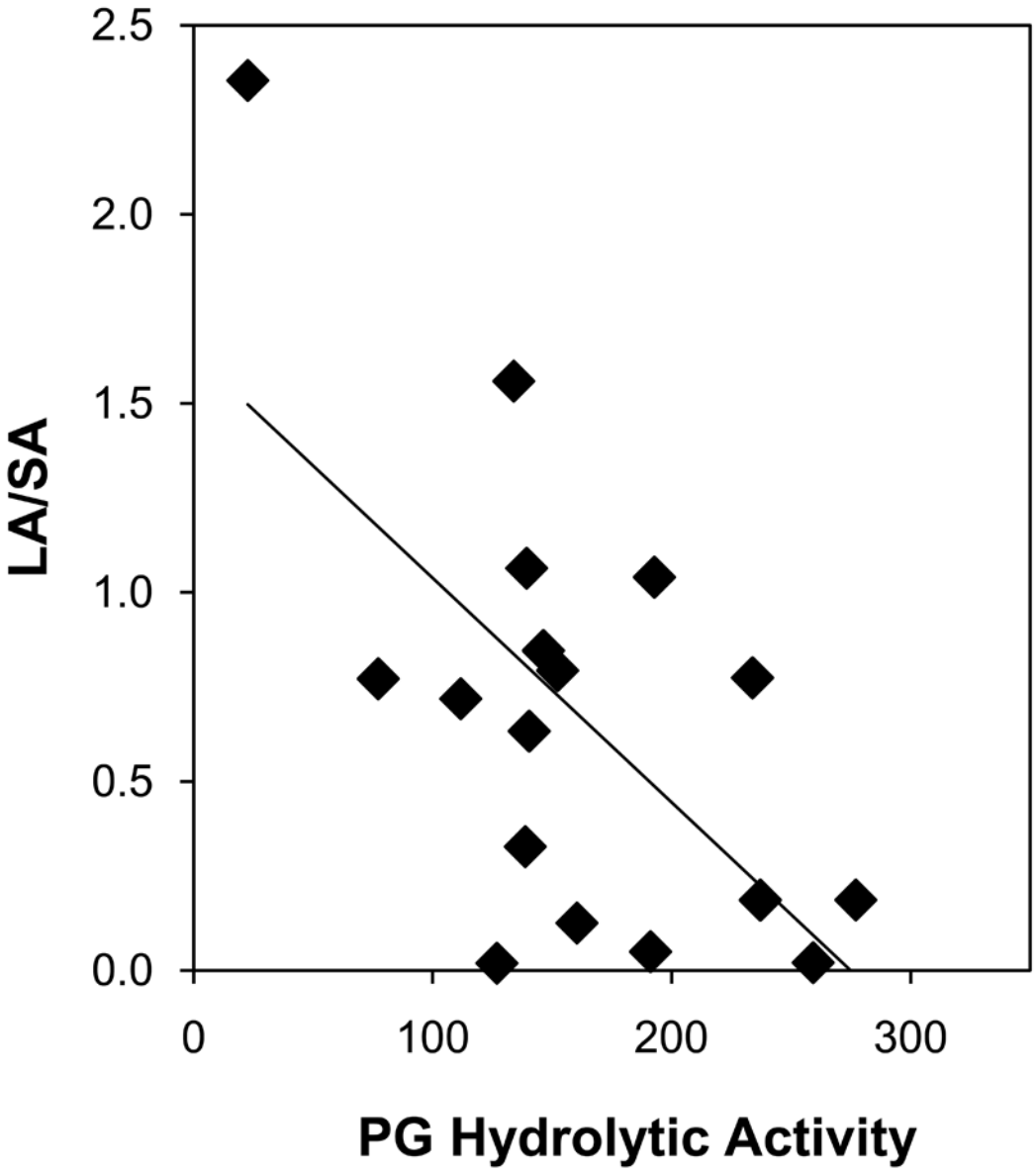

Figure 3. Change in surfactant LA/SA ratio is correlated with PG hydrolytic activity of $sPLA_2$ in the BAL sample

Phospholipid recovery was measured in the LA and SA fractions of ARDS and healthy control BAL samples and compared to $sPLA_2$ enzymatic activity ex vivo against PG. LA/SA ratios decreased with increasing $sPLA_2$ activity against PG in the ARDS BAL samples ($p < 0.01$, Pearson correlation, n=17).

Table 1

$sPLA_2$ Enzymatic Activity and Protein from ARDS vs Control BAL supernates.

$sPLA_2$ Measurement	**Healthy Controls**	**ARDS**
PG hydrolysis (pmol PL/sample)	16 (34)	143 (66) **
PC hydrolysis (pmol PL/sample)	31 (53)	88 (145) *
PLA2G2A protein (pg/ml)	28 (14)	696 (1353) **

Values for enzyme assays and EIA are as described in methods. All values are median (Interquartile range),

* designates $p \leq 0.05$ Control (n=10) vs ARDS (n=18),

** designates $p \leq 0.001$ Control vs ARDS

Table 2

Surfactant Aggregate Properties from ARDS vs Control BAL

Surfactant Quality	Healthy Controls	ARDS
γ min (mN/m)	0.3 (1)	23.9 (19.3) **
Large Aggregates (μg PL/ml)	17.3 (15.4)	17.9 (5.6)
Small Aggregates (μg PL/ml)	3.1 (4)	36.7 (61.4) **
Total Surfactant Phospholipid (μg PL/ml)	22.4 (9.5)	50.8 (91.5) *
Protein in SA (μg/ml)	62 (53)	1859 (2119) **
Protein in LA (μg/ml)	237 (21)	1148 (1817) **

Values in μg PL/ml or μg/ml are per ml of BAL fluid. All values are medians (Interquartile range).

* designates $p \leq 0.01$ Control (n=10) vs ARDS (n=17),

** designates $p \leq 0.001$ Control vs ARDS