

Antigen-induced Generation of Lyso-Phospholipids in Human Airways

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Summary

The goal of the current study was to examine the formation of phospholipids, 1-*sn*-glycero-3-phospholipids (lyso-PL) and 2-acetylated phospholipids (such as PAF) as well as mechanisms responsible for generating these phospholipids in bronchoalveolar lavage fluid (BALF) from allergic subjects challenged with antigen. Bronchoalveolar lavage was performed in normal and allergic subjects before, 5–30 min, 6 h, and 20 h after segmental antigen challenge via a wedged bronchoscope. Levels of 1-hexadecyl-2-lyso-phospholipids and 1-hexadecyl-2-acetyl-phospholipids were initially determined by negative ion chemical ionization gas chromatography/mass spectrometry (NICI-GC/MS). Antigen dramatically elevated quantities of 1-hexadecyl-2-lyso-phospholipids in allergic subjects 20 h after challenge when compared to non-allergic controls. In contrast, there was not a significant increase in levels of 1-hexadecyl-2-acetyl-phospholipids after antigen challenge. Closer examination of 1-*sn*-glycero-3-phosphocholine (GPC) revealed that 1-palmitoyl-2-lyso-GPC, 1-myristoyl-2-lyso-GPC and 1-hexadecyl-2-lyso-GPC were three major molecular species produced after antigen challenge. 1-palmitoyl-2-lyso-GPC increased sevenfold to levels of 222 ± 75 ng/ml of BALF 20 h after antigen challenge. The elevated levels of lyso-PL correlated with levels of albumin used to assess plasma exudation induced by allergen challenge. In contrast, the time course of prostaglandin D₂ (PGD₂) or 9 α , 11 β PGF₂ (11 β PGF₂) formation did not correlate with lyso-PL generation.

To examine the mechanism leading to lyso-phospholipid formation in antigen-challenged allergic subjects, secretory phospholipase A₂ (PLA₂) and acetyl hydrolase activities were measured. There was a significant increase in PLA₂ activity found in BALF of allergic subjects challenged with antigen when compared to saline controls. This activity was neutralized by an antibody directed against low molecular mass, (14 kD) human synovial PLA₂ and dithiothreitol. Acetyl hydrolase activity also markedly increased in BALF obtained after antigen challenge. This study indicates that high levels of lyso-PLs are present in airways of allergic subjects challenged with antigen and provides evidence for two distinct mechanisms that could induce lyso-PL formation. Future studies will be necessary to determine the ramifications of these high levels of lyso-phospholipids on airway function.

Asthma is a complex disease characterized by increased airway responsiveness to diverse stimuli and manifested by airway narrowing and inflammation (for review see reference 1). Evidence indicates that airway inflammation in asthma is characterized by an influx of inflammatory cells into airways and the release of inflammatory mediators, including biologically active phospholipids. One family of 2-acetylated phospholipids, collectively known as

platelet-activating factor (PAF)¹, is released by inflammatory cells *in vitro* in response antigenic and nonantigenic

¹Abbreviations used in this paper: BAL, bronchoalveolar lavage; BALF, BAL fluid; DTT, dithiothreitol; FEV₁, forced expiratory volume in 1 s; GPC, 1-*sn*-glycero-3-phosphocholine; HG, hydrofluoric acid; lyso-PC, lyso-phosphatidylcholine; PAF, platelet-activating factor; PFB, pentafluorobenzoyl; PLA₂, phospholipase A₂; PNU, protein nitrogen units; TLC, thin layer chromatography.

challenge (2, 3). Demonstrated biological activities of PAF *in vitro* suggest that the release of PAF *in vivo* into the human airway may modulate certain characteristics of asthma such as bronchoconstriction, cellular infiltration, mucous hypersecretion, microvascular leakage, and bronchial hyperreactivity (4–16).

Lyso-phospholipids are another family of phospholipids which have been proposed to regulate tissue injury. Although this phospholipid family has received a little attention in lung injury, it has been recognized for some time that the heart generates large quantities of lyso-phospholipids during injury such as myocardial ischemia (17–20). Once formed, these lyso-phospholipids have been hypothesized to contribute to electrophysiologic alterations that lead to arrhythmogenesis (18, 19, 21–28). A few investigations in animal models have also suggested that lyso-phospholipids can participate in lung injury by increasing lung resistance, inducing capillary permeability and altering surfactant properties (29–31). However, the formation of large quantities of lyso-phospholipids in response to injury has not been clearly demonstrated in the human lung.

Lyso-phospholipids can be generated in tissues by several different mechanisms. The most direct mode of synthesis is the hydrolysis of long chain fatty acids from the sn-2 position of membrane glycerolipids of tissues (32, 33). A family of phospholipase A₂ enzymes of relatively low molecular mass (~14 kD in size) classified as group I, group II and group III phospholipase A₂ (PLA₂) has been isolated as extracellular enzymes (for review see reference 34). In addition to their small sizes, they have high disulfide bond content and require millimolar calcium for catalysis. One member of this family, group II PLA₂, has been implicated in the pathogenesis of many inflammatory diseases (35–38). This enzyme has been shown to be released from several cells including mast cells during immunologic challenge (39, 40). Group II PLA₂ is present in high quantities in inflammatory sites such as peritoneal exudates from caseinate-treated animals, synovial fluids from patients with rheumatoid arthritis, and nasal lavage fluids from atopic subjects challenged with antigen (41–44). In addition, circulating levels of secretory PLA₂ increase in several illnesses including sepsis, shock, severe injury, and pancreatitis (45, 46).

A more indirect pathway that can generate lyso-phospholipids is the hydrolysis of the acetyl moiety at the sn-2 position of 2-acetylated phospholipids that are generated during tissue injury. Both serum and cellular acetyl hydrolase have been described as enzymes that are clearly distinct from PLA₂ isotypes (47–49). Recent studies by Tjoelker and colleagues suggest that the quantities of acetyl hydrolase that are found in tissues dramatically influence the response of that tissue to PAF (50).

In the current study, we examine the *in vivo* formation of phospholipid products, lyso-phospholipids, and PAF, from the PLA₂ and/or acetyl hydrolase reactions in airways of allergic individuals challenged with antigen. This study indicates that high levels of lyso-phospholipids as well as secretory PLA₂ and acetyl hydrolase are present in antigen-

stimulated airways. Furthermore, they raise fundamental questions as to the role of lyso-phospholipids in lung injury.

Materials and Methods

Study Population. Before the evaluation of subjects, protocols received Institutional Review Board approval, and informed consent was obtained. Four normal non-allergic, non-asthmatic subjects and three allergic asthmatic subjects underwent bronchoscopy and baseline bronchoalveolar lavage (BAL) without antigen challenge. 26 allergic subjects (9 with rhinitis alone and 17 with asthma and rhinitis) underwent segmental antigen challenge via a wedged bronchoscope and were lavaged at various time intervals (from 5 min to 20 h) following challenge. Bronchoalveolar lavage fluid (BALF) were assayed for 2-acetylated phospholipids and lyso-phospholipids. Subjects were men and women between 20 and 33 yr of age with no history of smoking. Subjects were classified as allergic asthmatics on the basis of clinical criteria, methacholine reactivity, and immediate-type skin testing as previously described (51). All asthmatic subjects reported a history of episodic wheezing, shortness of breath or chest tightness, and demonstrated a positive methacholine challenge test, defined as a 20% fall in the forced expiratory volume in 1 s (FEV₁) to a methacholine concentration <25 mg/ml. Allergic rhinitic subjects had a history of asthmatic symptoms and no response to methacholine at concentrations of 25 mg/ml. Atopic status was determined by prick-puncture skin testing with a battery of nine common inhalant allergens. All allergic subjects had two or more positive skin test reactions and were responsive to ragweed, Timothy grass, or dust mite used for segmental allergen challenge. All normal subjects had no history of asthma, were unresponsive to methacholine, and had no positive skin test reactions. All subjects had a FEV₁ >80% predicted, were asymptomatic, and required no medications for control of symptoms at the time of the study. No subject had a history of viral respiratory tract infection during the month before the study.

Bronchoalveolar Lavage and Segmental Antigen Challenge. Bronchoalveolar lavage was performed using a standardized protocol in accordance with guidelines established by a National Institutes of Health Workshop as previously described (52). Subjects were premedicated with atropine (0.6 mg IM). After inhalation of nebulized lidocaine (4%), an Olympus BF-10 fiberoptic bronchoscope was inserted into the airway. Local anesthesia was supplemented with lidocaine (2%) instilled via the bronchoscope. The bronchoscope was wedged in a subsegmental bronchus and a control sham challenge with 5 ml normal saline was performed. Antigen challenge was then performed by instilling 5 ml of antigen in a subsegment of the contralateral lung. Instillation of saline was always performed first to avoid contamination of the control subsegment by residual antigen via the bronchoscope. In this manner, each subject provided responses to saline control and to antigen. Most subjects were challenged with a total dose of 500 protein nitrogen units (PNU) of antigen, either short ragweed (*Ambrosia elatior*; Greer Laboratories, Inc., Lenoir, NC), dust mite (*Dermatophagoides farinae*; Hollister-Stier, Miles Inc., Elkart, IN), or Timothy grass (*Phleum pratense*; Hollister-Stier, Miles). 1 PNU/ml contained 0.005 µg/ml of ragweed antigen E. The endotoxin concentration of a 100 PNU/ml ragweed dilution was 0.05 endotoxin units measured using a colorimetric Limulus lysate assay. Bronchoalveolar lavage was performed by instilling 5 × 20-ml aliquots of normal saline, preheated to 37°C, with immediate aspiration of each aliquot. Fluids returned from each segment were

processed separately for mediator measurements. BALF recovery ranged from 45–60%. Upon recovery, BALF were pooled and filtered through gauze. Aliquots were immediately acidified with 8.8% formic acid (20 μ l/ml) to inactivate acid-labile acetylhydrolase. Fluids were then centrifuged (10 min, 400 g) to remove cells. 10 ng of the 1-0-16,16,16-trideuterohexadecyl-2-acetyl-GPC analogue of PAF (Biomol, Plymouth Meeting, PA), and two nanograms of 1-0-7,7,8,8-tetradeuterohexadecyl-2-lyso-1-radyl-2-lyso-sn-glycero-3-phosphocholine (GPC), a tetradeuterated analogue of lyso-PAF (Cayman Chemical, Ann Arbor, MI), were added to 2-ml aliquots of the cell-free supernatant fluids before sample extraction and derivatization procedures. BALF with internal standards were extracted and derivatized immediately or were stored at -70°C for subsequent extraction and derivatization.

Quantification of 2-acetylated Phospholipids and Lyso-phospholipids in BALF. BALF samples were extracted, and the pentafluorobenzoyl ester derivatives of 2-acetylated and lyso-phospholipids were synthesized and quantitated by combined capillary gas chromatography-mass spectrometry using modifications of methods previously described (53, 54). Briefly, 2-ml aliquots of BALF obtained and treated as described above were extracted with octadecylsilane cartridges (C18, Sep-Pak) (Waters Associates, Milford, MA) (55). Cartridges were conditioned with 10 ml of HPLC grade methanol (Fisher Chemical Co., Fairlawn, NJ) followed by 10 ml of HPLC grade water (Baker Chemical Co., Phillipsburg, NJ). 4 ml of methanol (Fisher Chemical Co.) and 4 ml of HPLC grade water (Fisher Chemical Co.) were added to 2-ml aliquots of acidified BALF containing the deuterated internal standards, creating a 40% (vol/vol) methanol/water mixture. The 40% methanol/water mixture was then loaded directly onto the conditioned cartridges, and the cartridge washed with 5 ml of water. The effluents from the loading and washing procedures were discarded. Phospholipid fractions containing 2-acetylated- and lyso-phospholipids were eluted from the cartridges with 5 ml of methanol and collected in Teflon-coated Oak-Ridge centrifuge tubes (Nalgene Co., Rochester, NY). The eluting solvent and residual water were evaporated under a nitrogen stream leaving a residue containing the eluted phospholipids. To remove the phosphobase moiety of extracted phospholipids, the residue was treated with 500 μ l of 49% hydrofluoric acid (HF) (Fisher Chemical Co.) and 1 ml hexane (Fisher Chemical Co.) with continuous shaking for 4 h at room temperature. The upper hexane layer was then removed, transferred to a clean silanized glass vial and dried under a stream of nitrogen. The pentafluorobenzoyl (PFB) esters of the resulting diglycerides were then synthesized by the addition of PFB esterification reagent. The pentafluorobenzoyl esterification reagent was prepared as follows: methylene chloride (Fisher Chemical Co.) was dried by passage of the solvent over anhydrous sodium sulfate (Aldrich Chemical Co., Milwaukee, WI). 2 mg of dimethylaminopyridine (Aldrich Chemical Co.) was then added to 600 μ l of anhydrous methylene chloride, followed by the addition of 4 μ l of pentafluorobenzoyl chloride (Aldrich Chemical Co.). 50 μ l of the resultant PFB esterification reagent was then placed onto the dried hexane residue and heated at 60°C for 5 min. The reaction solvent was evaporated under nitrogen, and the solid residue extracted twice with 0.5 ml of hexane and transferred to a clean silanized glass vial. The hexane-extracted product was then evaporated under nitrogen and dissolved in 30 μ l of dodecane (Aldrich Chemical Co.) for injection into the capillary gas chromatograph. The recovery of analytes during solid-phase extraction and derivatization procedures was determined by the addition of radiolabeled [^3H]PAF and [^3H]lyso-PAF (100,000 cpm) to

each BALF. Recovery of HF hydrolysis products of [^3H]PAF and [^3H]lyso-PAF was 95%. The overall yield throughout the extraction and esterification procedures was $>60\%$ (data not shown).

GC/MS analysis of derivatized phospholipids extracted from BALF was performed with a Varian 3400 GC interfaced with a Finnigan MAT TSQ-700 GC/MS/MS/DS operated as a single stage quadrupole. The mass spectrometer was operated in the negative ion chemical ionization mode using methane (Ultra High Purity; Matheson Gas Products, Bridgeport, NJ) at a pressure of 0.8–1.0 torr as the reagent gas. An emission current of 200 mA with an electron energy of 70V was employed for ionization. Characteristic molecular anions at m/z 552 (PAF) and 555 ($^2\text{H}_3$ -PAF) were monitored for measurement of *in vivo* PAF levels. 1-hexadecyl-2-lyso-phospholipid levels were determined by monitoring of the characteristic molecular anion for the bispentafluorobenzoyl ester (PFBOE) $_2$ of the monoglyceride (obtained after HF treatment) at m/z 704 using $^2\text{H}_4$ -lyso-PAF [signal at m/z 708] as an internal standard.

In a separate series of studies, levels of 1-myristoyl-2-lyso-GPC, 1-hexadecyl-2-lyso-GPC and 1-palmitoyl-2-lyso-GPC in BALF from seven atopic subjects obtained 20 h after antigen and saline challenges were determined. $^2\text{H}_4$ -1-Hexadecyl-2-lyso-GPC (5 ng) served as the internal standard for the three lyso-phosphatidylcholines (lyso-PC) and was added to BALF before solid-phase extraction. A lyso-phosphatidylcholine fraction was obtained via thin-layer chromatography before HF hydrolysis of the polar head group. Briefly, the methanol eluate from solid-phase extraction was dried, dissolved in 100 μ l of methanol and spotted onto silica gel G TLC plates and developed in a chloroform/methanol/acetic acid/water (50:28:8:3, vol/vol/vol/vol) solvent system. The resolved lyso-PC fraction of the TLC plate was scraped and extracted (3 \times) with 5-ml vol of methanol/water (3:1, vol/vol). The dried extract was then treated with HF for hydrolysis of the polar head group as described above. The monoglycerides generated via HF treatment of the lyso-phospholipids were extracted with hexane and transferred to a clean silanized glass vial. After solvent removal under a nitrogen stream, the monoglycerides were treated with pentafluorobenzoyl chloride in the presence of 4-dimethylaminopyridine and prepared for analysis as described above. The (PFBOE) $_2$ derivative of the monoglycerides were detected by monitoring molecular anions at m/z 690, m/z 704, m/z 718, and m/z 708, for elution of 1-myristoyl-(PFBOE) $_2$, 1-0-hexadecyl-(PFBOE) $_2$, 1-palmitoyl-(PFBOE) $_2$ and $^2\text{H}_4$ 1-0-hexadecyl-(PFBOE) $_2$, respectively. Unfortunately, we were unable to measure concentrations of 1-stearoyl-2-lyso-GPC in BALF by GC/MS because of a major contamination peak found in BALF that migrated with the 1-stearoyl-(PFBOE) $_2$.

Prostaglandin D_2 and Albumin Measurements in BALF. PGD $_2$ and its metabolite, 9 α ,11 β -PGF $_2$ (11 β -PGF $_2$), were assayed in BALF samples to determine if release of these inflammatory lipid mediators had been initiated following antigen challenge. Both prostaglandins were assayed by combined GC/MS using previously published methods (56). Limits of detection of the prostaglandins were 0.1–0.2 pg/ml BALF. Albumin was also assayed in lavage fluids and served as an index of airway permeability after segmental antigen challenge. Albumin was quantified using a sensitive radioimmunoassay (51).

Phospholipase A_2 Activity in BALF. BALF for PLA $_2$ activity measurements was obtained from five ragweed allergic, asthmatic subjects. These subjects were challenged with ragweed antigen at concentrations ranging between 20 and 500 PNU and were lavaged 20 h after challenge. Phospholipase A_2 activity in BALF was

measured as described by Fonteh and colleagues (40). Briefly, PLA₂ activity was determined in 50 mM Tris HCL (pH 7.4) and 10 mM CaCl₂ in a total volume of 1.0 ml. This reaction was initiated by the addition of 0.1 μCi (9.7 nmol) [³H]AA-labeled *Escherichia coli* membranes (New England Nuclear, Boston, MA). After incubation (90 min at 37°C) in a water bath, the reaction was stopped by extracting lipids by the method of Bligh and Dyer (40). Free fatty acids were isolated from phospholipids by TLC on silica gel G developed in hexane/ethyl ether/formic acid (90:60:6, vol/vol). The radioactivity in lipids was located by using a radiochromatogram imaging system (Bioscan Inc., Washington, DC). Free AA and phospholipids were isolated by TLC zonal scrapping and the quantity of radioactivity determined by liquid scintillation counting. PLA₂ activity was calculated and expressed as pmol AA released/h/ml of BALF.

In some experiments, the effects of antibody (F10-mAb) neutralization and dithiothreitol (DTT) on PLA₂ activity were examined. Portions of BALF were incubated (1 h, 37°C) with different concentrations of neutralizing antibody (F10-mAb) raised against recombinant human synovial fluid PLA₂. The antibody was a generous gift of Dr. Lisa Marshall (SmithKline Beecham, King of Prussia, PA). PLA₂ activity was determined in supernatant fluid fractions as described above. In other experiments, the effect of DTT on BALF PLA₂ activity was examined. Briefly, BALF was incubated with DTT on ice for 15 min. Subsequently, PLA₂ activity was measured as described above.

Acetyl Hydrolase Activity in BALF. The metabolism of PAF in BALF obtained from four allergic asthmatic subjects before and after antigen challenge was determined to ascertain acetyl hydrolase levels. [³H]PAF (1 μCi, 5 pmol; New England Nuclear, Boston, MA) was added (complexed to albumin [0.25 mg/ml]) to 5-ml aliquots of BALF obtained before, 5–30 min and 20 h after segmental antigen challenge via a wedged bronchoscope. BALF were maintained at 37°C and 1-ml aliquots of fluid were removed at 0, 10, 30, 60, and 90 min. Reactions were terminated by Bligh-Dyer extraction. The extracted phospholipid fractions were subjected to thin layer chromatography (TLC) using silica gel G plates with chloroform/methanol/acetic acid/water (50:28:8:3, vol:vol) as the developing system. The rate of metabolism of PAF to its [³H]lyso-PAF was determined by assessing the distribution of the radiolabel into the [³H]PAF and [³H]lyso-PAF fractions by liquid scintillation spectrometry.

Statistical Analysis. Statistical analysis between groups was made using Student's paired *t*-test. Correlations were assessed using Spearman rank correlation analysis.

Results

Formation of 2-acetylated Phospholipids and Lyso-phospholipids in Human Airways. Initial experiments were designed to examine the quantities of PAF and closely related 2-acetylated analogs (i.e., 1-hexadecyl-2-acetyl-phospholipids) as well as lyso-phospholipids containing an 1-hexadecyl-linkage at the sn-1 position of the glycerol backbone in BALF fluids. Levels of 1-hexadecyl-2-acetyl-phospholipids as determined by GC/MS are depicted in Fig. 1. Quantities of 2-acetylated phospholipids in allergic subjects were near or below the detection limits (50 pg/ml) of the assay before antigen challenge. These values were not significantly different from those found in non-allergic subjects (data not shown). Antigen challenge did not significantly increase

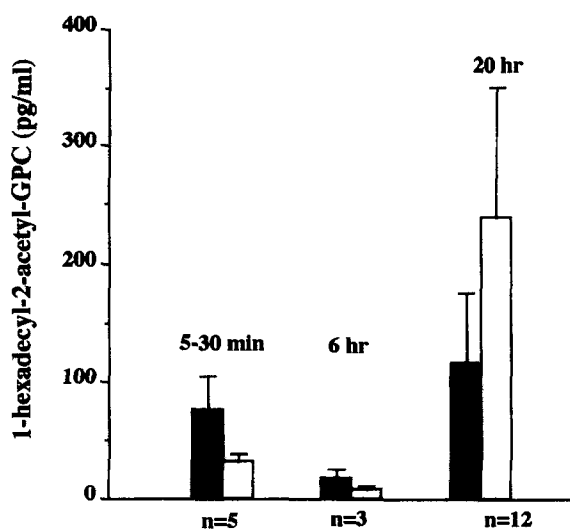


Figure 1. 1-Hexadecyl-2-acetyl-phospholipid levels before and after antigen challenge. BALFs were obtained at the indicated times after antigen challenge. Internal standards were added and lipids extracted. 2-Acetylated phospholipids were converted to pentafluorobenzoyl derivatives and analyzed by NICI-GC/MS. 1-hexadecyl-2-acetyl-phospholipid levels in the absence and presence of antigen are shown. These data are the means ± SEM. ■, Normal saline; □, antigen.

quantities of 2-acetylated phospholipids in BALF of allergic subjects 5–30 min, 6 or 20 h after challenge. We next measured the quantities of 1-hexadecyl-2-lyso-phospholipids in BALF of allergic subjects before challenge and found 22 ± 0.9 ng/ml, a value 20–30-fold higher than 2-acetylated phospholipids. These levels were not significantly different from those found in nonallergic subjects (data not shown).

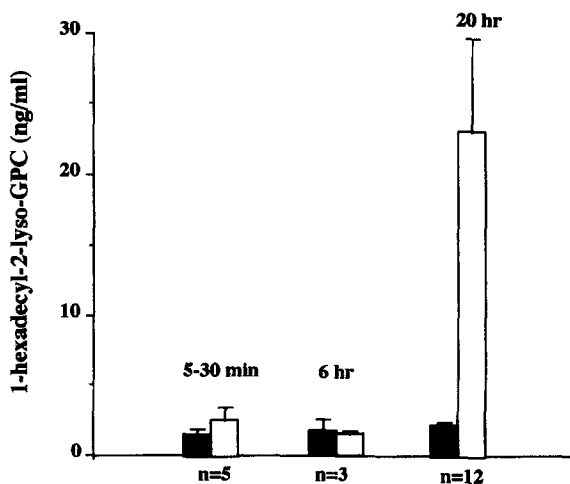


Figure 2. 1-Hexadecyl-2-lyso-phospholipid levels before and after antigen challenge. BALFs were obtained at the indicated times after antigen challenge. Internal standards were added and lipids extracted. Lyso-phospholipids were converted to bispentafluorobenzoyl derivatives and analyzed by NICI-GC/MS. 1-hexadecyl-2-lyso-phospholipid levels in the absence and presence of antigen are shown. These data are the means ± SEM. ■, Normal saline; □, antigen. **P* < 0.05.

Data in Fig. 2 illustrates that there was no significant increase in quantities of 1-hexadecyl-2-lyso-phospholipids in BALF of allergic subjects at 5–30 min and 6 h after antigen challenge when compared to saline challenge. However, levels of 1-hexadecyl-2-lyso-phospholipid were significantly increased (compared to saline challenge or baseline levels) 20 h after antigen challenge ($P < 0.05$).

While these initial studies measured one select subclass of lyso-phospholipid, 1-hexadecyl-2-lyso-phospholipid, they provided no data regarding other lyso-phospholipid molecular species that may be produced during antigen challenge. To address this question, 1-acyl-2-lyso-GPC molecular species were isolated by TLC and analyzed by NICI-GC/MS. Fig. 3 illustrates the GC/MS analysis of several lyso-PC molecular species found in the BALF of seven allergic subjects 20 h after antigen challenge. Levels of 1-palmitoyl-2-lyso-GPC, 1-myristoyl-2-lyso-GPC and 1-hexadecyl-2-lyso-GPC were increased five- to ninefold in allergic subjects challenged for 20 h with antigen when compared to saline challenge controls. In particular, levels of 1-palmitoyl-2-lyso-GPC were high ($222 \text{ ng/ml} \pm 75$) in the late phase BALF. Fig. 4 illustrates the individual levels of 1-palmitoyl-2-lyso-GPC in seven subjects. While 1-palmitoyl-2-lyso-GPC levels increased in the BALF of all subjects after antigen challenge, the degree of change varied a great deal between individual subjects. The level of 1-palmitoyl-2-lyso-GPC in the BALF of one subject were extremely high approaching 700 ng/ml.

Phospholipase A_2 Activity in Human Airways. To gain some understanding into the mechanism(s) leading to the marked increases in 1-acyl-2-lyso-GPC molecular species in BALF, the activities of two potential enzymes that could regulate

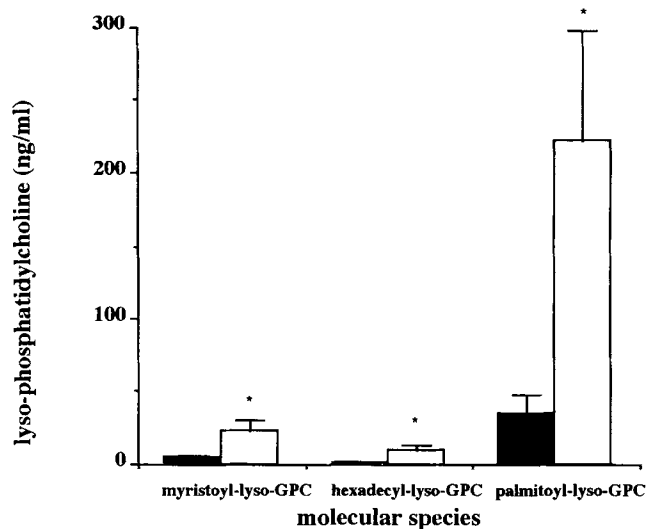


Figure 3. Influence of 20 h antigen challenge on levels of lyso-phosphatidylcholine molecular species. BALFs were obtained 20 h after antigen challenge. Lipids were extracted and lyso-phosphatidylcholine isolated by TLC. Lyso-phosphatidylcholine molecular species were then converted to bispenta-fluorobenzoyl derivatives and analyzed by NICI-GC/MS. These data are the means \pm SEM of seven separate patients. ■, Normal saline; □, antigen. * $P < 0.05$.

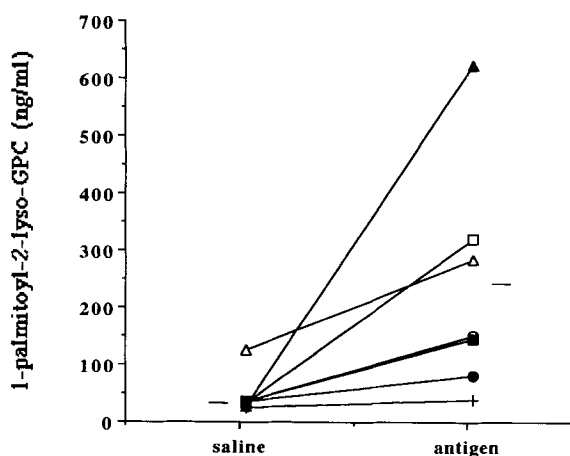


Figure 4. Influence of antigen challenge on levels of 1-palmitoyl-2-lyso-GPC in seven allergic subjects. BALFs were obtained 20 h after antigen challenge. Lipids were extracted and lyso-phosphatidylcholine isolated by TLC. Lyso-phospholipids were then converted to bispentafluorobenzoyl derivatives and analyzed by NICI-GC/MS. These are the individual measurements of 1-palmitoyl-2-lyso-GPC from seven separate patients.

lyso-phospholipids levels were measured. PLA₂ activity was assayed by measuring the capacity of cell-free BALF to hydrolyze [³H]AA from labeled *E. coli* membranes. Fig. 5 shows the PLA₂ activity in BALF from five allergic subjects challenged for 20 h with saline or antigen. BALF from saline challenged sites contained relatively high levels of PLA₂ activity ($981 \pm 121 \text{ pmol/h/ml BALF}$). In addition, there was a modest and significant increase in PLA₂ activity found in BALF after antigen challenge when compared to saline challenge. PLA₂ activity increased after antigen challenge in the BALF of all five subjects tested.

To better characterize this PLA₂ activity, BALF from allergic subjects (saline and antigen challenged) were exposed to either a neutralizing antibody (F10) directed against low

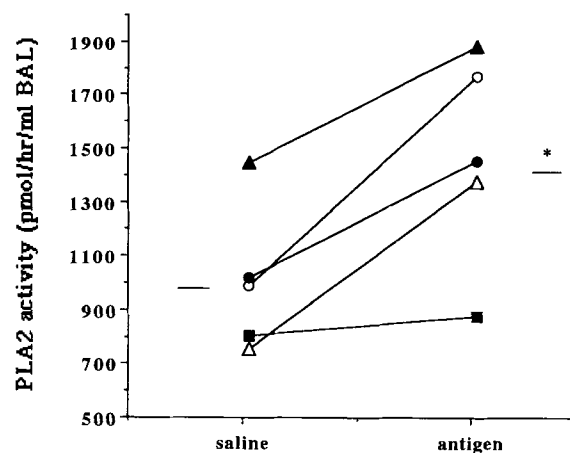


Figure 5. PLA₂ activity before and after antigen challenge. BALFs were obtained 20 h after antigen challenge. PLA₂ activity was determined using labeled *E. coli* membranes as described in the methods. These are the measurements from five patients challenged with saline and antigen. * $P < 0.05$.

molecular secretory PLA₂, or a reducing agent, dithiothreitol (Fig. 6, A and B). Both the neutralizing antibody and dithiothreitol blocked the activity of PLA₂ in BALF in a dose-dependent manner. These data reveal that the BALF of allergic subjects contain high levels of PLA₂ activity with characteristics of low molecular weight, group II PLA₂.

Acetyl Hydrolase Activity in Human Airways. An increase in lyso-phospholipid levels could also result from the hydrolysis of an acetyl moiety from the sn-2 position of the glycerol backbone. Both cellular and serum acetyl hydrolase activities which are distinct from classical PLA₂ have been described (47-50). Acetyl hydrolase activity was determined in BALF by monitoring the catabolism of [³H]PAF. [³H]PAF was added to BALF recovered before and 5-30 min and 20 h after antigen challenge. Fig. 7 indicates that acetyl hydrolase activity in BALF varies significantly depending on

whether the antigenic response has been initiated. Top, middle, and bottom panels illustrate acetyl hydrolase activity before, 5-30 min and 20 h after antigen challenge. Whereas acetyl hydrolase activity is present before antigen

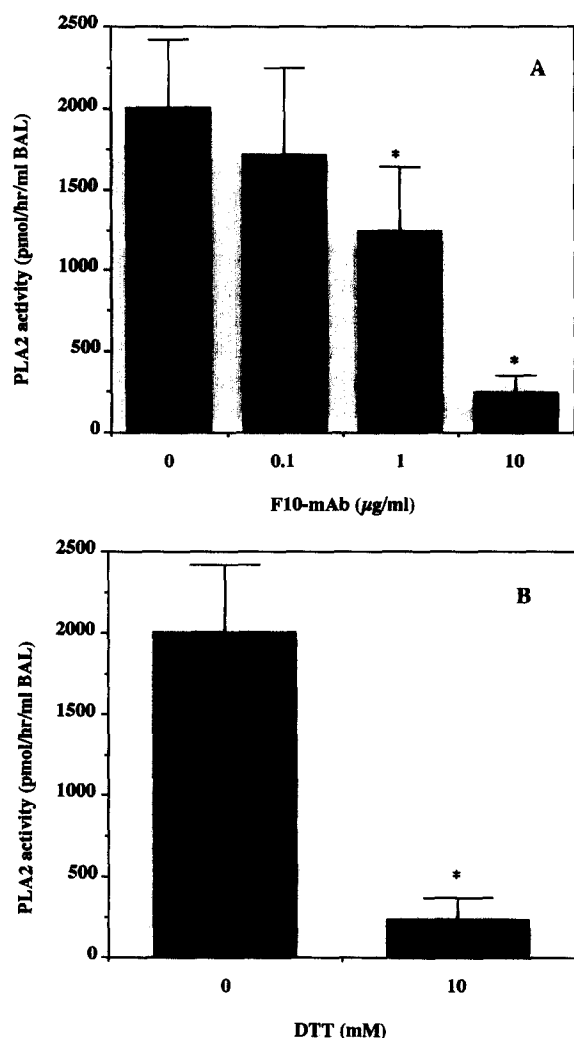


Figure 6. Effect of F-10 antibody and dithiothreitol on BALF PLA₂ activity. BALFs were obtained 20 h after antigen challenge. The fluids were treated with an antibody (F10) that neutralizes human synovial PLA₂ and DTT. PLA₂ activity was determined using labeled *E. coli* membranes as described in the methods. These are the measurements from three patients challenged with antigen. **P* < 0.05.

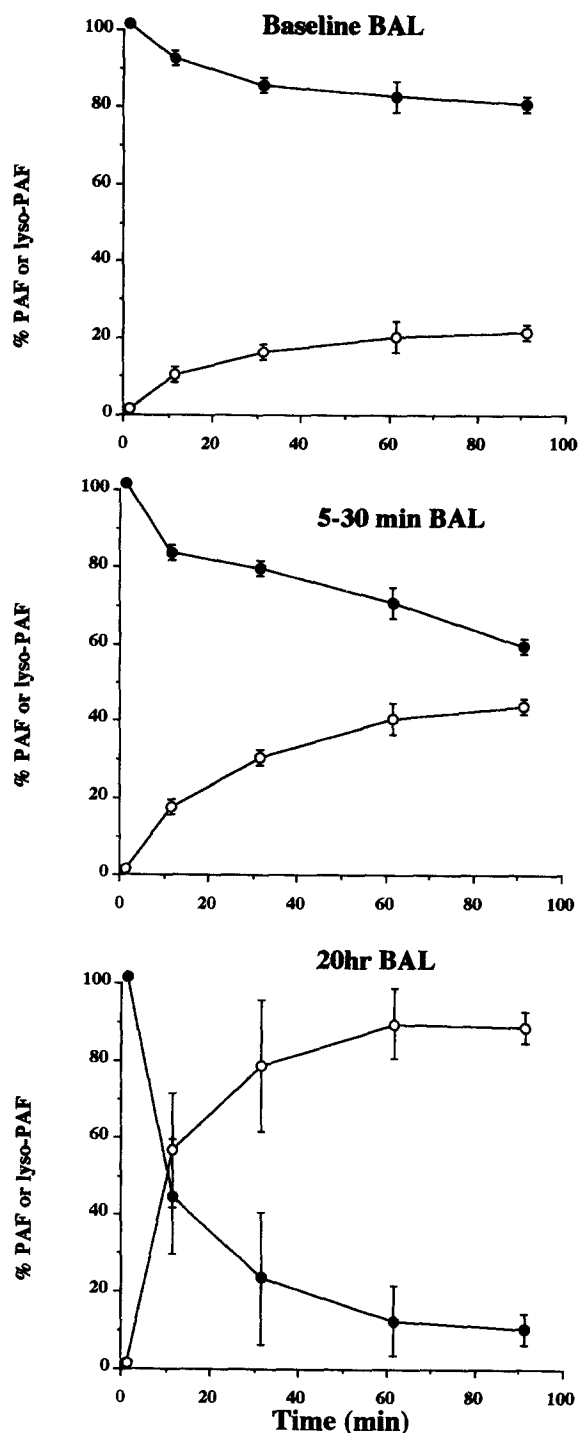


Figure 7. Acetyl hydrolase activity in BALF before and after antigen challenge. BALFs were obtained at the indicated times after antigen challenge. Labeled PAF was incubated in BALF and metabolites were isolated by TLC. These data show the conversion of PAF to lyso-PAF and are means \pm SEM of four patients at each time point. ●, PAF; ○, lyso-PAF.

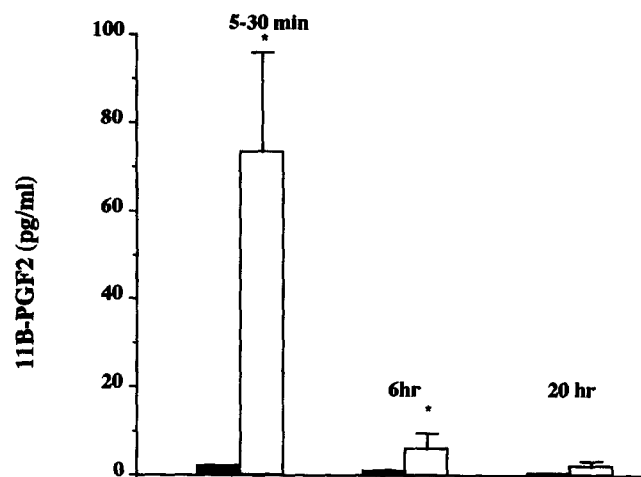
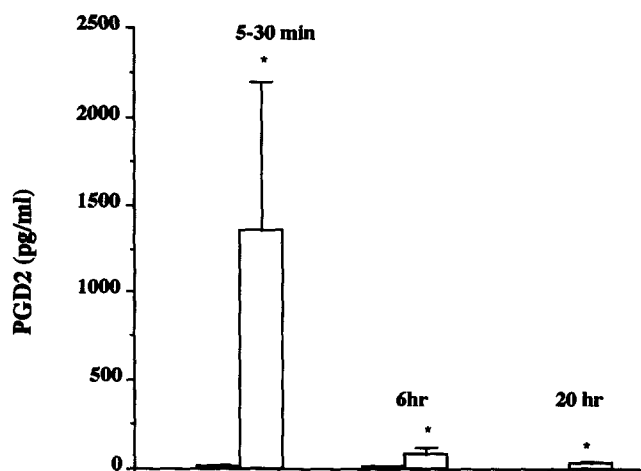


Figure 8. PGD₂ and 11βPGF₂ levels before and after antigen challenge. BALFs were obtained at the indicated times after antigen challenge. Internal standards were added and lipids were extracted. Prostaglandins were derivatized as described and analyzed by NICI-GC/MS. PGD₂ and 11βPGF₂ levels in the absence and presence of antigen are shown. These data are means ± SEM. ■, Normal saline; □, antigen. *P < 0.05.

challenge, there is a time-dependent increase in BALF acetyl hydrolase activity after antigen challenge. At 20 h after antigen challenge, >80% of added PAF is hydrolyzed within 30 min.

Correlation of Lysophospholipid Levels with Changes in Prostaglandin D₂ and Albumin in Human Airways. The next series of experiments were designed to examine whether changes in lysophospholipid levels could be correlated with other markers of antigen activation. Levels of PGD₂ and its metabolite 11β-PGF₂ were measured by NICI-GC/MS in BALF obtained before, 5–30 min, 6 and 20 h after antigen challenge. Levels of both PGD₂ and 11β-PGF₂ increased

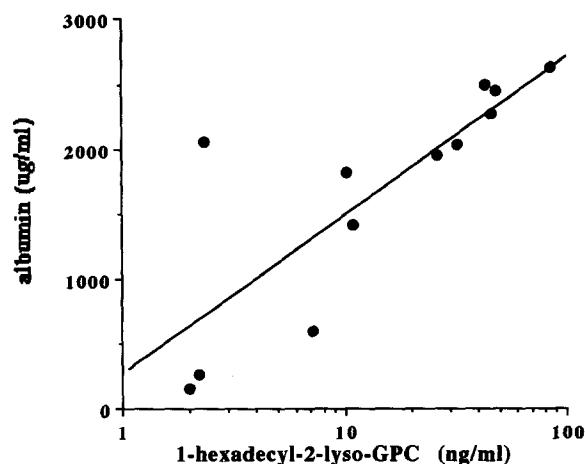


Figure 9. Correlation between 1-hexadecyl-2-lyso-phospholipids and albumin levels. BALFs were obtained 20 h after antigen challenge. Internal standards were added to an aliquot of the BALF and lipids extracted. Lyso-phospholipids were converted to bispentafluorobenzoyl derivatives and analyzed by NICI-GC/MS. Albumin levels were determined in another aliquot as described in the methods. P = 0.004 by Spearman Rank correlation.

significantly 5–30 min (Fig. 8) after challenge when compared to saline challenge levels. PGD₂ levels were much lower but remained slightly elevated over saline challenge levels at 6 and 20 h. These data demonstrated that the time course of formation of PGD₂ and 11βPGF₂ was clearly different than that seen for lyso-phospholipids. In contrast to these prostaglandins, albumin levels were markedly elevated 20 h after antigen challenge (Fig. 9). When these levels were compared to levels of 1-hexadecyl-2-lyso-phospholipids in individual subjects, there was a significant correlation between albumin and 1-hexadecyl-2-lyso-phospholipid levels.

Discussion

Studies in a variety of tissues and the heart, in particular, have shown that high concentrations of lyso-phospholipids are generated during injury. This can be demonstrated in vitro with isolated myocytes, in models of ischemia-perfusion injury, or in vivo during myocardial infarction. Whereas the exact role of these lyso-phospholipids in heart injury is not clear, it is known that they can contribute to membrane dysfunction, electrophysiological alterations, lethal arrhythmias, and cellular injury of the ischemic myocardium. In contrast to the heart, there have been few studies examining the role of lyso-phospholipids in lung injury. This may be due, in large part, to the fact that it is difficult to measure lyso-phospholipids in tissues and that there have been few studies that have reported lyso-phospholipid formation at significant levels in the lung. It has been shown that measurable quantities of lyso-PAF are present in BALF of both normal, rhinitic and asthmatic subjects (12, 57). Results in the current study reveal that relatively high concentration of lyso-phospholipids reside in the epithelial lining fluid of allergic subjects and levels dramatically increase during

challenge with antigen. Whereas all potential molecular species of lyso-phospholipids were not examined, these data indicate that at least three major molecular species, 1-myristoyl-2-lyso-GPC, 1-hexadecyl-2-lyso-GPC and 1-palmitoyl-2-lyso-GPC are found in BALF of human airways.

At least two fundamental questions arise from these data: (a) what are the roles of these high concentrations of lyso-phospholipids in lung injury? and (b) what are the enzymatic mechanism(s) which generate lyso-phospholipids in human airways? In regard to the former, the role of lyso-phospholipids in lung injury has not been clearly elucidated. However, mean BALF concentrations of 1-palmitoyl-2-lyso-GPC at 20 h were 222 ng/ml and reached levels of 685 ng/ml in one subject. Since the amount of saline injected into the bronchus could dilute the alveolar lining fluid from 50- to 100-fold (58), the mean concentration of 1-palmitoyl-2-lyso-GPC in lining fluid may range from 25 to 50 μ M after antigen challenge and could reach 150 μ M in some subjects. Lyso-phospholipids levels this high affect several biological processes that could alter lung function. These concentrations are 10–20-fold higher than the critical micelle (aggregate) concentration for lyso-PC, and this physical form of lyso-phospholipids can disrupt cellular membranes via detergent effects resulting in cytolysis. For example, PLA₂-induced lyso-PC formation in isolated hamster lungs has been correlated with type I epithelial cell toxicity (29, 33). In addition to detergent effects, lyso-PC can irreversibly inactivate surfactant (30) by its capacity to disorder the surface film thereby reducing the ability to lower surface tension. Lyso-phospholipids have also been shown to increase airway and capillary permeability in isolated perfused rat lungs (31). The fact that albumin concentrations increase as a function of the concentration of lyso-phospholipids in BALF further emphasizes the potential role of lyso-phospholipid in inducing cell injury and changes in airway permeability. There is also some question as to whether the lyso-phospholipids could serve as precursors for the biosynthesis of 2-acetylated phospholipids such as PAF. For example, we have demonstrated that activated human neutrophils can readily utilize exogenously-provided 1-hexadecyl-2-lyso-GPC for the synthesis of 1-hexadecyl-2-acetyl-GPC (59). Finally, very recent studies have shown that relatively low concentrations of lyso-PC transcriptionally regulate growth factor gene expression in human monocytes and endothelial cells (60, 61). Taken together, these data imply that lyso-phospholipids have great potential to affect lung injury.

In contrast to the potential deleterious impact of lyso-phospholipids, these lyso-phospholipids may represent key intermediates in pathways for generating new surfactant or for membrane lipid remodeling. Alternatively, several studies indicate that polyunsaturated acyl residues of phospholipids can undergo oxidative damage and fragmentation in the presence of free radicals generated by activated inflammatory cells (for a review see reference 62). Watson and colleagues have further demonstrated that acetyl hydrolase has the capacity to cleave many of these oxidatively fragmented phospholipids to form lyso-phospholipids (63).

Therefore, lyso-phospholipids observed in our studies may represent the product of clearance of oxidatively damaged phospholipids by lipases such as acetyl hydrolase.

With regard to the question of mechanism of formation, again it is difficult to unequivocally predict how lyso-phospholipids are formed during antigen challenge. Acute respiratory failure associated with disorders such as pancreatitis correlates with serum levels of PLA₂ (46). We recently demonstrated that nasal lavage of allergic subjects contains PLA₂ activity and lyso-phospholipids and that both are enhanced markedly following nasal challenge with antigen or histamine (43, 57). The nasal lavage PLA₂ was purified and found to be similar to a recombinant form of human PLA₂ identified in synovial fluids from patients with arthritis (43). The current study shows that a secretory PLA₂ is present in BALF of normal and allergic subjects and that this activity was increased significantly in allergic subjects after antigen challenge. This activity was also blocked by a neutralizing antibody directed against synovial fluid PLA₂ and dithiothreitol, consistent with characteristics of a low molecular mass secretory PLA₂. Although there is no direct evidence that secretory PLA₂ contributes to lyso-phospholipid generation in human airways, group I PLA₂ has been shown to induce lyso-phospholipid formation in hamster lung preparations (33). Other studies have demonstrated that mast cells contain group II PLA₂ and release this enzyme during the antigen-induced cross-linking of IgE (39, 40). Therefore, it may be that human mast cells release secretory PLA₂s which, in turn, hydrolyze lung phospholipids to form lyso-phospholipids.

The lung source of the phospholipid substrate for the PLA₂ reaction is unknown. In the hamster lung, surfactant phospholipids were suggested to be the preferred substrate for secretory PLA₂. As mentioned above, PLA₂ has been shown to destroy the activity of isolated lung surfactant and this loss of activity has been postulated to be due to the formation of lyso-phospholipids. Interestingly, the ratio of 1-myristoyl-2-lyso-GPC to 1-palmitoyl-2-lyso-GPC observed in these studies correlates well with the 1-myristoyl-2-palmitoyl-GPC to 1-palmitoyl-2-palmitoyl-GPC ratio in human lung surfactant. However, the presence of relatively large amounts 1-hexadecyl-2-lyso-GPC suggest that cellular phospholipids in the lung may also serve as a substrate for secreted PLA₂. Group I and Group II PLA₂ can interact with various cells through specific receptors on their plasma membranes (64, 65) and this may account for high levels of other lysophospholipids in BALF of antigen-challenge individuals.

As mentioned above, it is also possible that lyso-phospholipids are formed by the hydrolysis of 2-acetylated or oxidatively damaged phospholipids released into the lung. This hypothesis is supported by the fact that acetyl hydrolase levels in BALF increase in a time-dependent manner following antigen challenge. However, two lines of reasoning argue against acetyl hydrolase generating the bulk of the observed lyso-phospholipids. First, the ratios of 1-myristoyl-, 1-palmitoyl- and 1-hexadecyl-2-lyso-GPC, do not fit the pattern of 2-acetylated phospholipids thought to be

synthesized during antigen activation in the lung. For example, previous studies suggest little 1-myristoyl-2-acetyl-GPC and more 1-hexadecyl-2-acetyl-GPC are synthesized by mast cells within the lung (66). Second, high levels of 2-acetylated phospholipids such as PAF would be required in airways to serve as a substrate for acetyl hydrolase. This would seem unlikely since we were unable to measure levels much higher than 50 pg/ml of BALF at any time point after antigen challenge. However, this does not rule out the possibility that large quantities of oxidatively-damaged phospholipids are formed during antigen challenge and lyso-phospholipids observed in the late phase are a result, in part,

of acetyl hydrolase-catalyzed cleavage of these phospholipids.

In conclusion, lyso-phosphatidylcholine levels are markedly elevated in BALF obtained 20 h after antigen challenge from allergic subjects. The elevated lyso-phospholipids are likely a result of elevated secretory PLA₂ or acetyl hydrolase levels. This antigen-dependent accumulation of lyso-phospholipids in the lung may play an important role in the disrupted airways permeability, barrier function and surfactant properties characteristic of late inflammatory responses.

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