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Lysophosphatidic acid is detectable in human bronchoalveolar lavage fluids at baseline and increased after segmental allergen challenge

S. N. Georas^{**}, E. Berdyshev^{†*}, W. Hubbard[‡], I. A. Gorshkova[†], P. V. Usatyuk[†], B. Saatian^{*}, A. C. Myers^{*}, M. A. Williams^{*}, H. Q. Xiao^{*}, M. Liu^{*} and V. Natarajan[†]

^{*}Divisions of Pulmonary & Critical Care Medicine and Allergy & Clinical Immunology, The Johns Hopkins Asthma & Allergy Center, Baltimore, MD, USA, [†]Division of Pulmonary & Critical Care Medicine, University of Chicago, Chicago, IL, USA and [‡]Department of Clinical Pharmacology, Johns Hopkins University, Baltimore, MD, USA

Clinical and Experimental Allergy

Summary

Background Lysophosphatidic acid (LPA) is a biologically active lysophospholipid and a component of normal plasma. LPA binds to receptors expressed on circulating and structural lung cells and affects cell growth and activation. Whether LPA is present in the lung has not been previously reported.

Objective To develop an assay to measure LPA in bronchoalveolar lavage (BAL) fluids, and to study the association between LPA and allergic airway inflammation.

Methods Seventeen allergic subjects underwent bronchoscopy and segmental allergen challenge, followed 18 h later by BAL. Supernatants were analysed for LPA content using liquid chromatography and mass spectroscopy. Expression of LPA receptors on primary bronchial epithelial cells was analysed by immunolabelling, and the effects of LPA on epithelial cell barrier function was investigated by measuring transepithelial resistance.

Results LPA was detectable in BAL from control lung segments, and significantly increased 18 h after allergen challenge. Polyunsaturated species of LPA were especially increased following segmental allergen challenge. LPA levels did not strongly correlate with the number or percentages of eosinophils, neutrophils or lymphocytes, whereas MIP-3 α (CCL20) levels correlated significantly with the allergen-driven influx of lymphocytes. The levels of LPA from control sites correlated inversely with BAL protein content, suggesting that LPA promoted epithelial barrier integrity at baseline. Experiments using primary human bronchial epithelial cells confirmed that LPA tightened the epithelial cell barrier.

Conclusion Lysophosphatidic acid is detectable in human BAL fluids at baseline and its expression increases during allergic inflammation. LPA does not appear to be a dominant chemoattractant for eosinophils or lymphocytes during allergic airway inflammation. In the absence of ongoing inflammation, LPA may promote epithelial barrier integrity.

Keywords allergic inflammation, asthma, barrier integrity, chemokine, epithelial cell, fatty acid, lysophosphatidic acid, mass spectrometry

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Correspondence:

Steve N. Georas, University of Rochester Medical Center, Rochester, NY, USA.

E-mail:

Steve_Georas@urmc.rochester.edu

Introduction

Biologically active lysophospholipids are generated by many types of mammalian cells and exert powerful effects on cell growth and gene expression in different tissues (for review see [1]). Lysophosphatidic acid (LPA) is one of the most prominent members of the glycerophospholipid family of signaling molecules. LPA is present in plasma

where it is bound to proteins (e.g. albumin) that enhance its delivery to target cells, especially in injured tissues [2, 3]. LPA can be generated through multiple metabolic pathways. During *de novo* phospholipid biosynthesis, LPA serves as an intermediate in the synthesis of complex phospholipids. In contrast, LPA generated through catabolic transformation of complex glycerophospholipids can have a direct signalling function. The hydrolysis of lysophosphatidyl choline (LPC) by lysophospholipase D, recently identified as autotaxin [4, 5], is one of the major

*These authors contributed equally to this work.

pathways leading to the generation of extracellular and bioactive LPA. LPC (and other substrates for this reaction) are provided by membrane microvesicles from platelets and other cells [6, 7].

LPA has been well characterized as a growth factor because it promotes the survival and inhibits apoptosis of numerous cell types including smooth muscle cells [8], endothelial cells [9], lymphocytes [10], and epithelial cells [11]. In fact, LPA is one of the major serum growth factors [12]. LPA was recently linked to neointima formation during atherogenesis reflecting its ability to induce vascular remodelling [13] and platelet activation [14]. LPA can act on several cells types of potential relevance to lung diseases [15]. Toews and colleagues established that LPA is a mitogen and spasmogen for airway smooth muscle cells that acts in concert with other growth factors [8, 16, 17]. These studies implicated LPA as a bronchoconstrictor of potential relevance to asthma, but whether and how LPA gains access to airway smooth muscle cells in asthma is currently unclear. LPA enhanced the recruitment of eosinophils and neutrophils to the guinea-pig lung [18], and also promoted chemotaxis of human eosinophils *in vitro* [19]. Furthermore, LPA can augment the secretion of the neutrophil chemoattractant IL-8 and other cytokines from bronchial epithelial cells [20, 21]. Because both eosinophils and neutrophils are enriched in the asthmatic lung, these data suggest that LPA may play a role in airway inflammation either directly (acting as a chemoattractant) or indirectly (by promoting chemokine production).

In contrast to these potentially pro-inflammatory effects, LPA might have a role in homeostasis and promote the resolution of inflammation. For example, LPA decreases the permeability of endothelial cells and augments barrier function [22, 23]. In addition, LPA suppressed the basal production of some cytokines by bronchial epithelial cell lines [21]. In a topical application model, LPA promoted healing of skin wounds in rats *in vivo* [24]. Thus the net effect of LPA will depend on when and where it is produced during an inflammatory reaction, and how it impacts on its target cells.

The diverse biological effects of LPA are mediated by a widely expressed family of receptors that include G-protein coupled membrane receptors (GPCRs) and possibly other intracellular targets [25]. The LPA-binding GPCRs belong to the endothelial differentiation gene (Edg) receptor family, and when expressed on the cytoplasmic membrane bind extracellular LPA with nanomolar affinities (see [26] for review). A new nomenclature for the Edg receptor family was recently adopted which renamed the three Edg family members LPA₁, LPA₂, and LPA₃ (formerly Edg 2, Edg 4, and Edg 7, respectively) [27]. LPA receptors couple to different downstream signalling pathways and exert non-redundant effects on their target cells. For example, LPA₃ is required during mouse embry-

ogenesis [28], whereas LPA₁ is essential for post-natal development of the nervous system [29]. A fourth and structurally distinct LPA receptor, distantly related to the purinergic receptor family, was recently identified [30]. In addition to these GPCRs, LPA can bind to other intracellular targets including the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPAR- γ [31] and mechano-gated potassium channels [32], possibly in a cell-type-specific manner. In contrast to LPA-GPCR interactions, the structural basis of interactions between LPA and its other targets is not as well understood.

Previous studies from our group and others showed that late-phase allergic reactions are characterized by pronounced influx of inflammatory cells into the airway as well as striking increases in BAL phospholipids and lysophospholipids [33, 34]. Allergen challenge also causes surfactant dysfunction in part by altering its phospholipid composition [35]. However, there is currently little information about whether LPA is detectable in the human lung and if it is associated with lung inflammation. Here we report that LPA is constitutively present in human bronchoalveolar lavage (BAL) fluids, and increased following segmental allergen challenge. The content and molecular species composition of LPA present in BAL fluids were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). Here we show that LPA levels were increased during allergic inflammation, especially polyunsaturated species, but that LPA levels correlated only weakly with inflammatory cell influx.

Methods

Materials

Standards of LPA with different fatty acid chains (17:0 as internal standard, 18:0, 18:1, 18:2, and 20:4, where the first number indicates the number of carbon atoms and the second number indicates the number of double bonds) were from avanti polar lipids (Alabaster, AL, USA). For *in vitro* studies of epithelial barrier function, LPA (18:1) was purchased from avanti polar lipids and sonicated immediately before use to ensure uniform dispersion. ELISA kits to measure the content of MIP-3 α and IL-8 and BAL were from R&D system (Minneapolis, MN, USA).

Bronchoscopy with segmental allergen challenge and bronchoalveolar lavage

We identified 17 subjects who had previously undergone allergen challenge for whom stored BAL samples were available for analysis. The demographics of subjects and other data are indicated in Table 1. All BAL samples were stored at -80°C until analysis and had not undergone

Table 1. Subject demographics

BAL number	Age (Years)	Sex	Allergen
997	42	m	RW
1015	36	m	RW
1041	19	f	RW
1056	27	m	RW
1065	27	m	RW
1081	27	m	DPT
1275	33	m	DPT
1296*	35	f	DPT
1333	22	f	DPT
861	21	f	RW
877*	25	f	RW
880*	33	f	RW
964*	35	m	RW
976*	45	f	RW
988*	32	m	RW
1067*	37	m	DF
1074*	28	f	RW

*Eight subjects for whom LPA species were analysed in Fig. 4.

BAL, bronchoalveolar lavage; m, male; f, female; RW, ragweed; DPT, *Dermatophagoides pteronyssinus*; DF, *Dermatophagoides farinae*.

freeze-thaw cycles. The method of segmental allergen challenge involves introduction of allergen or saline control into the lung via a wedged bronchoscope followed 18 h later by BAL. We used an IRB-approved protocol as previously reported [36]. BAL cell pellets were analysed for total cell count using a haemocytometer, and cell differential was determined after staining with Diff Quick (Pierce, Rockford, IL, USA). At least 300 cells were counted per sample. BAL supernatants were analysed for total protein content using a colorimetric method (Pierce), and IL-8 and MIP-3 α levels in BAL were determined using commercially available ELISA kits according to the manufacturer's instructions.

Lipid extraction

A fraction of BAL samples (0.5 mL) were acidified with 12 N HCl to have a final concentration of 0.2 N HCl and BAL lipids were extracted with *n*-butanol (2 mL) by extensive vortexing. C17:0 LPA (162 pmol) was added to each sample as internal standard before vortexing. After phase separation by centrifugation at 1500 *g* for 5 min, *n*-butanol was collected and evaporated in a vacuum. The residue was dissolved in 0.3 mL methanol and subjected to LC-MS/MS analyses.

Analysis of LPA by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

LPA content and individual molecular species composition were measured by means of electrospray ionization MS/MS analysis after lipid separation by HPLC. An API-4000 triple quadrupole mass spectrometer (Applied Bio-

systems, Foster City, CA, USA) coupled with an Agilent 1100 liquid chromatography system (Agilent Technologies, Foster City, CA, USA) was used for the study. Lipids were separated on Zorbax eclipse XDB-C8 (Agilent Technologies, Foster City, CA, USA), 4.6 $\times$ 150 mm, 5 μ m using methanol/water/HCOOH, 79/20/0.5, v/v, with 5 mM NH₄COOH as solvent A and methanol/acetonitrile/HCOOH, 59/40/0.5, v/v, with 5 mM NH₄COOH as Solvent B. LPA molecular species were analysed in negative mode with declustering potential and collision energy optimized for 17:0, 18:0, 18:1, 18:2, and 20:4 LPA. An example of the linearity of the approach for 18:1 LPA is shown in Fig. 2a. Multiple reaction monitoring parameters for nine other LPA molecular species were selected with the closest possible approximation with available LPA standards. The following transitions were monitored: 407.0/153.0 (16:1 LPA); 409.0/153.1 (16:0 LPA); 423.0/153.1 (17:0 LPA (I.S.)); 431.0/153.0 (18:3 LPA); 433.0/153.0 (18:2 LPA); 435.1/152.9 (18:1 LPA); 437.0/153.0 (18:0 LPA); 455.1/153.0 (20:5 LPA); 457.0/153.0 (20:4 LPA); 459.1/153.0 (20:3 LPA); 461.1/153.0 (20:2 LPA); 481.1/153.0 (22:6 LPA); 483.1/153.0 (22:5 LPA); 485.1/153.0 (22:4 LPA).

Analysis of LPA receptor expression by immunohistochemistry

Lung tissue not needed for human transplantation was obtained from cadaveric donors as described [37]. The anonymous human tissue obtained in this manner has been determined by the Committee on Human Research of the Johns University to be exempt from human subjects review. Blocks from lungs containing visible airways were fixed in 4% formaldehyde, frozen, and sectioned (5 μ m). Immunohistochemical identification of LPA receptors was performed using antibodies directed against LPA₁ and LPA₂ (antibodies kindly provided by Drs. K. E. Belmonte and H. M. Sarau, GlaxoSmithKline, King of Prussia, PA, USA) or control rabbit IgG as described [38].

Analysis of LPA₁ surface expression on primary human bronchial epithelial cells by flow cytometry

The trachea and main bronchi were dissected from human lung tissues (obtained as above), and primary airway epithelial cells (PAEC) obtained following overnight digestion at 4 °C in 0.1% (w/v) protease Sigma type XIV (Sigma-Aldrich) as described [37]. Epithelial cells were washed and resuspended in staining buffer (PBS supplemented with 0.5% v/v normal mouse serum, 20 mM HEPES buffer pH 7.4 and 0.02%w/v sodium azide) at 10⁵ PAEC in 100 μ L of staining buffer. PAEC were incubated for 10 min with a supplement of 25 μ L normal mouse serum (Sigma-Aldrich) and 100 μ g/mL rabbit anti-mouse whole IgG (Sigma-Aldrich, St. Louis, MO, USA) in PBS to block Fc γ receptors. PAEC were next incubated for 30 min on ice with appropriate dilutions of anti-LPA1 receptor antibody

(a rabbit polyclonal N-terminal extracellular domain-specific antibody obtained from Lifespan Biosciences Inc., Seattle, WA, USA). Cells were washed twice in FACS staining buffer and resuspended in 100 μ L staining buffer. Next, cells were incubated for 10 min with a supplement of 25 μ L normal mouse serum (Sigma-Aldrich) and 100 μ g/mL rabbit anti-mouse whole IgG (Sigma-Aldrich) in PBS to block residual Fc γ receptors to which was added a 1/500 dilution of allophycocyanin-conjugated AffiniPure F(ab')₂ fragments of goat anti-rabbit IgG (Jackson Immuno Research Labs. Inc., West Grove, PA, USA). Cells were incubated on ice for 15 min, washed three times in FACS staining buffer and finally resuspended in 400 μ L staining buffer before flow cytometric analysis. A FACS Caliber flow cytometer (Becton Dickinson, San Diego, CA, USA) was used for data analysis using Cellquest software. Data were collected as geometric mean fluorescence intensity (MFI) and percent fluorescent positive cells in list mode.

Analysis of the effects of LPA on epithelial barrier function by transepithelial electrical resistance

In order to test the possibility that LPA may promote epithelial barrier integrity, primary passage epithelial cells were seeded on gelatin-coated gold electrodes (eight wells, one electrode per well) to $\sim$ 95% confluence, and placed into an electrical cell-substrate impedance sensing system (ECIS; Applied Biophysics Inc., Troy, NY, USA) incubator for 1 h to stabilize basal electrical resistance before treatment with LPA as indicated. The total epithelial electrical resistance, as measured across the monolayer, was determined by the combined resistance between the basal and/or cell matrix adhesion as described [39]. Measurements were done in triplicate and expressed as normalized resistance (means $\pm$ SD) for each of the treatments.

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. Significance between groups was analysed using paired Student's *t*-test or Wilcoxon' signed rank as indicated, with $P < 0.05$ considered to be statistically significant. The Spearman's correlation coefficient and its 95% confidence interval was computed using Microsoft Excel. Significance values of linear correlations were analysed using StatDisk 9.6.1.

Results

LPA is constitutively present in the lung but increased following allergen challenge

Table 1 shows the demographics of the 17 subjects who underwent allergen challenge. There were nine men and

eight women with an average age of 30.8 years. Sixteen subjects had allergic asthma and one had allergic rhinitis. All subjects underwent segmental allergen challenge using a wedged bronchoscope technique [36]. All subsequent data are reported as the average for the entire group of subjects: separate analyses of different subsets (e.g. male vs. female, ragweed vs. dust mite) did not affect any of the conclusions reported. As shown in Fig. 1, there was a substantial influx of cells into the airway following allergen challenge. The total number of recovered cells (calculated by multiplying the cell count per mL with the total volume of recovered lavage fluid) increased from 17.21 ± 2.11 to 87.39 ± 19.10 million cells, a $\sim$ 5-fold increase ($P < 0.005$). This was due to a large influx of eosinophils (from 0.10 ± 0.02 to $44.3 \pm 13.2 \times 10^6$ cells), with smaller increases in total lymphocyte and neutrophil numbers (from 2.07 ± 0.31 to 10.05 ± 2.25 and 2.00 ± 0.69 to $17.5 \pm 6.43 \times 10^6$ cells, respectively, Fig. 1a). When expressed as cell differential, there was a large relative increase in the percent of eosinophils, and a relative

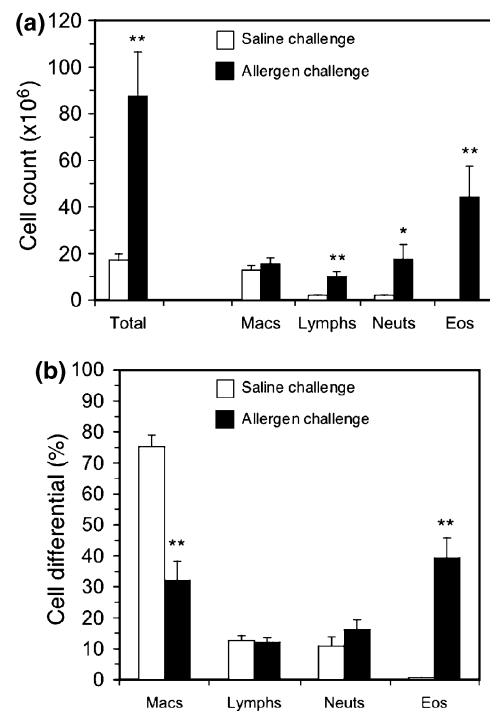


Fig. 1. Segmental allergen challenge results in bronchoalveolar lavage (BAL) eosinophilia. (a) The total number of cells recovered from allergen-challenged lung segments (black bars) was substantially higher than from saline-challenged control sites (open bars). This was due to the influx of eosinophils (Eos), neutrophils (neuts) and lymphocytes (lymphs). (b) The relative percentage of macrophages (mac) was reduced following allergen challenge, whereas lymphocytes were unchanged. The percentage of neutrophils increased slightly following allergen challenge (from 10.84 ± 3.09 to $16.25 \pm 3.12\%$, $P = 0.11$), whereas eosinophils increased substantially (from 0.61 ± 0.12 to $39.28 \pm 6.57\%$, $P < 0.005$). Data are the mean $\pm$ standard error of the mean (SEM) from 17 subjects; * $P \leq 0.05$ and ** $P \leq 0.005$ using the paired Student's *t*-test.

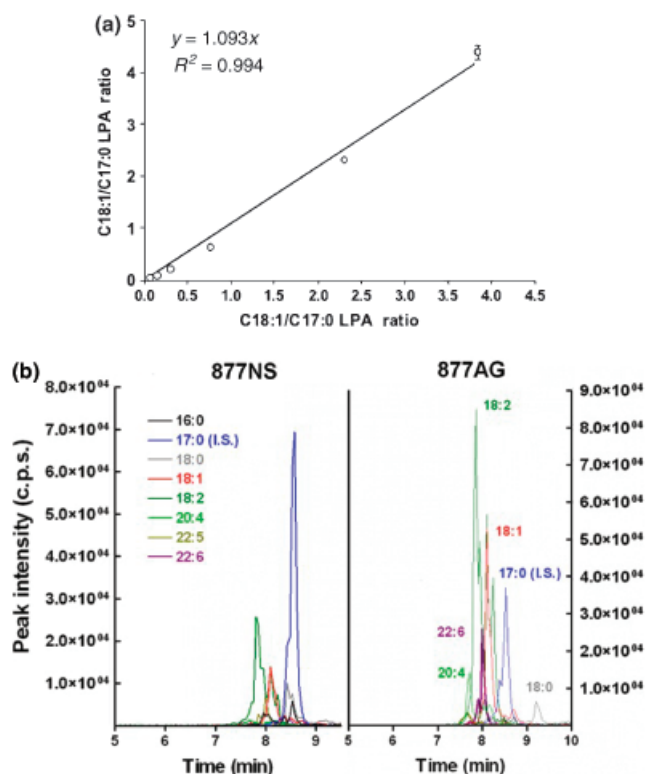


Fig. 2. (a) Linearity of MS/MS response for lysophosphatidic acid (LPA) standards. The increasing amount of 18:1 LPA was added to a fixed amount of 17:0 LPA (161.9 pmol). The discovered C18:1/C17:0 ratios are plotted against actual ratio values. (b) Representative example of LPA molecular species detected following challenge with normal saline (NS) or allergen (Ag).

decrease in the percent of macrophages, recovered from allergen challenge lung segments (Fig. 1b). These data are in keeping with our and others' prior experience with the human allergen challenge model [36, 40].

In order to measure LPA levels in BAL fluids, we developed a quantitative assay using LC-MS/MS with C17:0 LPA as an internal standard. After lipid extraction with *n*-butanol, LPA molecular species were separated by HPLC and then analysed using electrospray ionization and tandem mass spectrometry (see (Methods)). Figure 2 shows that this method results in a highly linear correlation ($R^2 = 0.9951$) when comparing observed vs. actual LPA ratios (Fig. 2a). Sample histograms from saline and allergen-challenged lung segments are shown in Fig. 2b. LPA was detectable in BAL samples from saline-challenged control lung segments, but that there was a significant ~3-fold increase in LPA following allergen challenge (from 483 ± 77 to 1506 ± 358 pmol/mL, $P = 0.0045$, mean $\pm$ SEM, $n = 17$). These data establish for the first time, to our knowledge, that the concentration of LPA is increased during allergic lung inflammation. The detection of LPA from saline-challenged control segments is consistent with prior findings showing that lysophospholipids are present constitutively in human BAL fluids [33].

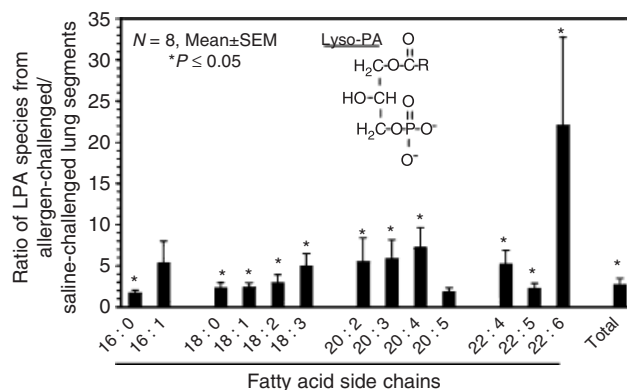


Fig. 3. Different lysophosphatidic acid (LPA) molecular species are selectively increased following allergen challenge. Data are the ratio of LPA fatty acids from allergen vs. saline challenged lung segments, and are the mean $\pm$ standard error of the mean (SEM) of $n = 8$ samples that were able to be fully analysed. $*P < 0.05$. Cartoon inset indicates structure of LPA with fatty acid (R) at the -1 position.

Tandem MS/MS with multiple reaction monitoring allowed us to determine the LPA molecular species expressed in the lung during allergic inflammation. Interestingly, this analysis revealed that certain polyunsaturated LPA molecular species were the most increased following segmental allergen challenge. As shown in Fig. 3, 22:6 (docosahexaenoic) LPA was increased the most, followed by eicosanoic species including 20:4-(arachidonyl)LPA. The comparably lower amounts of 16:0 LPA argue against surfactant-derived saturated phosphatidylcholine (PC), which is predominantly dipalmitoyl 16:0/16:0 PC [41], as a major source. Because polyunsaturated LPA species are usually generated from complex phospholipids as breakdown products by activated phospholipases, these data are consistent with catabolic LPA generation during lower airway inflammation (see (Discussion)).

There was individual heterogeneity in the LPA response. For example, although on average each of the LPA species increased, there were decreases in LPA species in a few individuals. In particular subjects 976 and 988 demonstrated on average an approximate 30% reduction in total LPA from allergen-challenged sites. Interestingly, in these two subjects certain LPA species were still elevated in BAL following allergen challenge, including a 3.4-fold increase in 16:1 LPA in subject 976 and a 22.5-fold increase in 22:6 LPA in subject 988. Furthermore, inspection of the 22:6 LPA values appeared to suggest the presence of five "responders" (demonstrating increases of 7.9-, 16.7-, 39.7-, 22.5- and 89.0-fold) and three "non-responders" (demonstrating decreases of 0.5-, 0.1-, and 0.1-fold) when comparing allergen vs. saline challenged lung segments. Although certain indices of inflammation were lower in the "non-responders" (e.g. total protein content and IL-8 values), the smaller numbers of subjects in the two groups precluded a meaningful subset analysis. The mechanisms

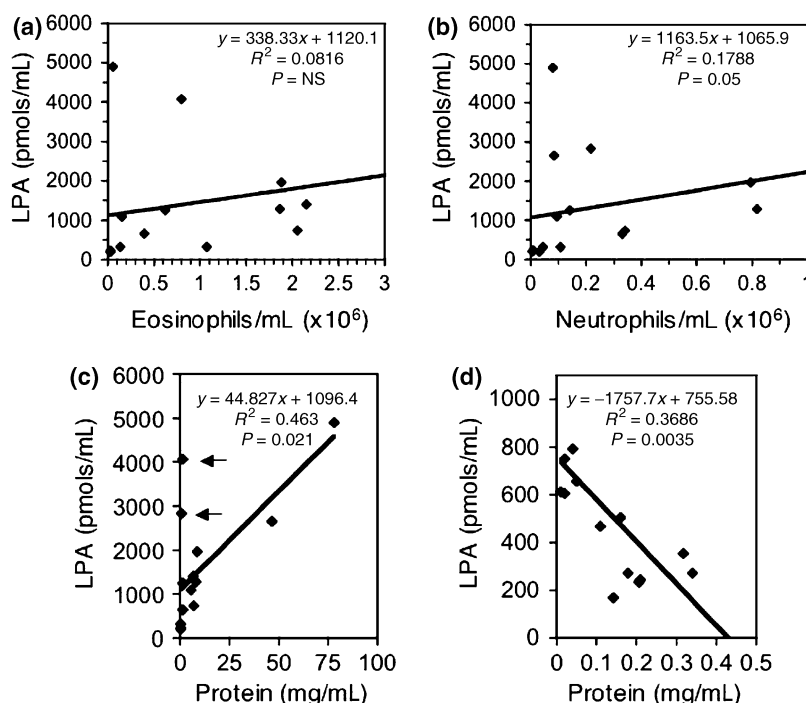


Fig. 4. Correlations between bronchoalveolar lavage (BAL) lysophosphatidic acid (LPA) levels, leucocyte numbers and protein content. The graphs indicate lack of correlation between LPA levels and (a) eosinophils, and weak correlations with (b) neutrophils, and (c) protein content from allergen-challenged lung segments. (d) LPA levels and protein content were inversely correlated in BAL from saline-challenged control sites. See text for details.

and significance of variability in LPA values and LPA species will need to be determined in future research.

We next determined whether LPA levels correlated with the influx of inflammatory cells or other parameters in BAL fluids from saline- or allergen-challenged lung segments. Interestingly, LPA levels did not correlate significantly with total cell count or the influx of eosinophils or lymphocytes following allergen challenge (Fig. 4a and data not shown). LPA levels correlated weakly with neutrophil numbers although there was substantial variability in this regard (Fig. 4b, $r = 0.43$, $P = 0.05$, 95% confidence interval -0.12 – 0.77). We measured total protein content in the BAL supernatants and found that this was increased following allergen challenge (0.16 ± 0.03 vs 10.4 ± 5.32 mg/mL from saline- vs. allergen-challenged lung segments, $P = 0.04$), consistent with prior reports [36]. There was a modest correlation between LPA and total protein levels from allergen-challenged lung segments (Fig. 4c, $r = 0.68$, $P = 0.021$, 95% confidence interval 0.26 to 0.89), although two subjects showed large increases in BAL LPA without substantial changes in protein (arrows, Fig. 4c). Interestingly, there was a highly significant negative correlation between LPA levels and protein content in BAL from saline-challenged control lung segments (Fig. 4d, $r = 0.61$, $P = 0.0035$, 95% confidence interval 0.14 – 0.85), which is considered further below.

We next investigated what other chemoattractants were associated with cell influx into the lung during the late-

phase allergic reaction. We focused on the chemokines IL-8 (CXC chemokine ligand 8 or CXCL8) and macrophage inflammatory protein-3 α (MIP-3 α , CC chemokine ligand 20, or CCL20) because these chemokines are produced by lung and airway cells and promote the recruitment of neutrophils (IL-8) and lymphocytes (MIP-3 α) [42–44], two cell types whose numbers were increased following allergen challenge (Fig. 1). Levels of both chemokines were increased following segmental allergen challenge (IL-8 from 15.9 ± 5.9 to 178.3 ± 110.3 pg/mL, and MIP-3 α from 13.3 ± 7.3 to 115.8 ± 34.3 pg/mL, following saline vs. allergen challenge, respectively, both $P < 0.01$). Interestingly, IL-8 levels did not correlate significantly with neutrophil numbers or percentages (data not shown). In contrast, we observed a strong correlation between the levels of MIP-3 α and total cell count and lymphocyte numbers recovered following allergen challenge (Figs 5a and b). We also observed weaker but significant correlations between MIP-3 α levels and the numbers of neutrophils and eosinophils (Figs 5c and d). Neither MIP-3 α nor IL-8 levels correlated with BAL LPA levels from either saline- or allergen-challenged sites (data not shown).

Because BAL protein content from control lung segments reflects in part the leak of vascular proteins including albumin into the lung and airways, the strong inverse correlation between BAL protein content and LPA levels from saline-challenged control lung segments (Fig. 4d) suggested that LPA may have a barrier protective role in

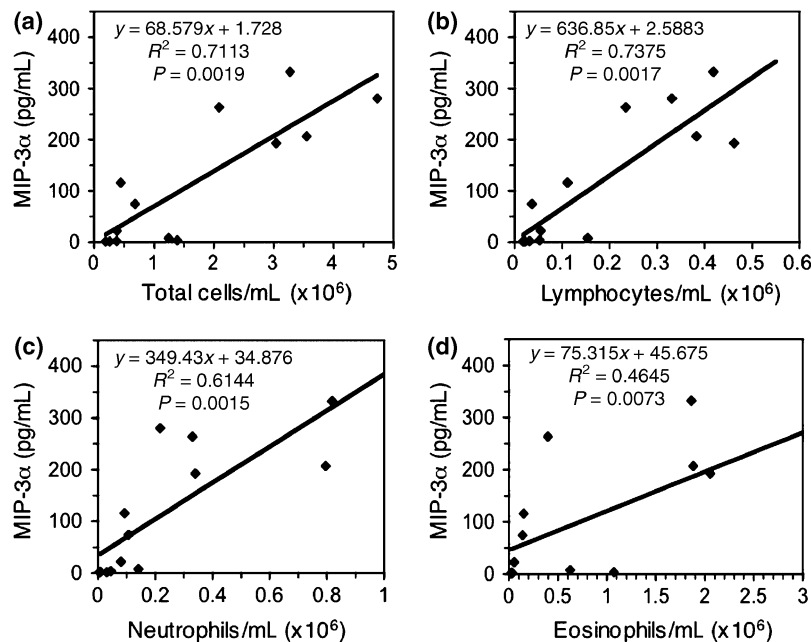


Fig. 5. Correlations between macrophage inflammatory protein (MIP)-3 α levels and leucocyte numbers in bronchoalveolar lavage (BAL) from allergen-challenged lung segments. The graphs indicate linear correlations between MIP-3 α levels and (a) total cells/mL ($r = 0.84$, $P = 0.0019$, 95% CI from 0.58 to 0.95), (b) lymphocytes/mL ($r = 0.86$, $P = 0.0017$, 95% CI from 0.62 to 0.95), (c) neutrophils/mL ($r = 0.78$, $P = 0.0015$, 95% CI from 0.45 to 0.92), and (d) eosinophils/mL ($r = 0.70$, $P = 0.0073$, 95% CI from 0.30 to 0.89).

the absence of lung inflammation. In order to investigate this possibility further, we cultured primary human bronchial airway epithelial cells *in vitro* and studied the effects of LPA on epithelial barrier function. We first wanted to confirm that airway epithelial cells used in these studies expressed receptors for LPA. Figure 6a shows that airway epithelial cells in human lung tissue express both LPA₁ and LPA₂ receptors as determined by immunohistochemistry. We were unable to obtain reproducible staining with low background using antibodies directed against LPA₃ in these experiments (data not shown). Most currently available LPA receptor antibodies are directed against intracellular domains and can not detect surface expression in intact cells. An LPA₁ receptor antibody directed against the conserved extracellular N-terminus was recently generated allowing for cell surface expression to be studied by immunofluorescence and flow cytometry (see (Methods)). Using this antibody, we found that the vast majority of primary human epithelial cells express cell surface LPA₁ (Fig. 6b). Specificity of staining was confirmed in studies using spleen cells from LPA₁ knock-out mice in which surface staining was essentially abrogated (M. Williams and S. Georas, unpublished observation). In order to investigate the effects of LPA on epithelial barrier function, primary passage cells were analysed using an electrical cell substrate impedance system in which increasing resistance indicates a tighter cellular barrier (see (Methods)). In these experiments, we found that LPA induced a significant and dose-dependent increase in resistance,

indicating that it promotes barrier function of the epithelial cell layer (Fig. 7).

Discussion

There are several novel observations contained in this report. First, we show that concentration of the bioactive lysophospholipid LPA is detectable at baseline but significantly increased in the lung after segmental allergen challenge. This is due primarily to increases in polyunsaturated molecular species of LPA. However, LPA BAL levels did not significantly correlate with BAL cell counts or the influx of eosinophils into the lung after segmental allergen challenge. In contrast, we found that the chemokine MIP-3 α was strongly correlated with the influx of total leucocytes including lymphocytes. At baseline, LPA may exert a barrier protective effect because it promotes epithelial barrier function in the absence of ongoing inflammation. These and other observations will be considered further below.

Because of its ability to promote the growth, differentiation, and activation of numerous cell types, there is growing interest in the ability of LPA to regulate immune and inflammatory reactions. Several lines of evidence indicate that LPA may play a role in the pathogenesis of asthma. First, LPA can act as a smooth muscle mitogen and spasmogen [8, 16, 17]. Second, in animal models or *in vitro* studies, LPA was shown to promote the activation and/or recruitment of eosinophils and neutrophils

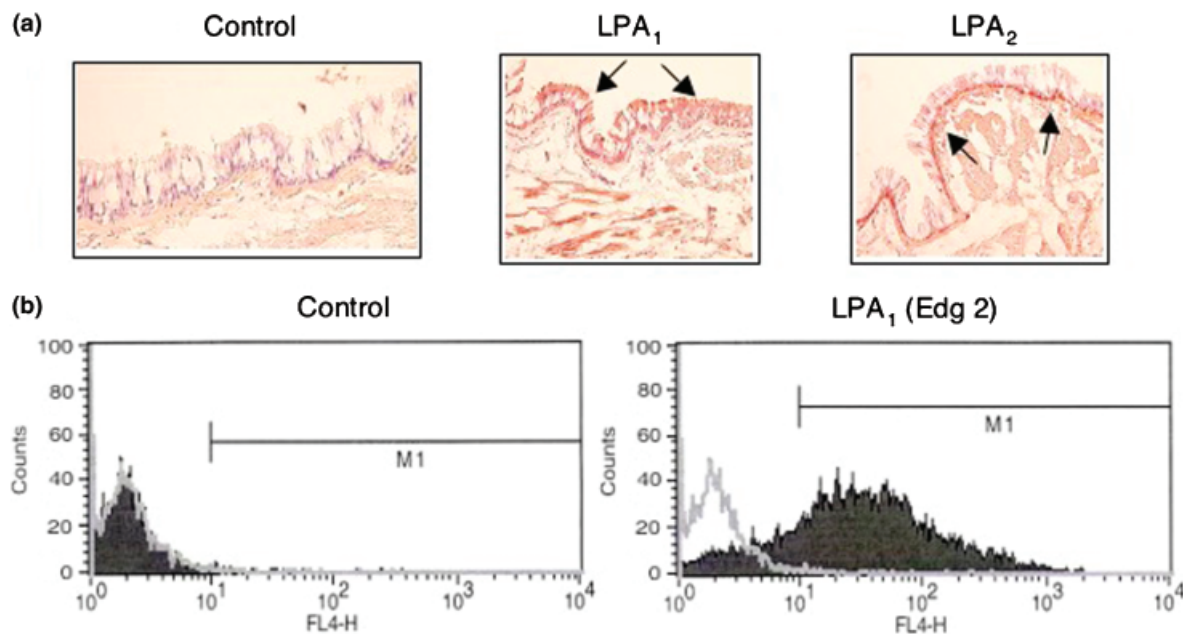


Fig. 6. Expression of lysophosphatidic acid (LPA) receptors by human airway epithelial cells. (a) Five micron sections of human lung tissue was analysed by immunohistochemistry using antibodies directed against LPA₁ (Edg2) and LPA₂ (Edg4) or rabbit IgG (control) as indicated. Note the pronounced apical expression of LPA₁ (arrows, middle panel), whereas LPA₂ expression was prominent on the basal surface (arrows, right panel). The figure depicts lung tissue from adjacent sections from one lung, representative of three different lungs from healthy subjects with no known lung disease. (b) Cell surface expression of LPA₁ was confirmed on dispersed primary bronchial epithelial cells by immunofluorescence and flow cytometry. Cells were prepared as described in *Materials and Methods* and assessed for percent positivity and mean channel fluorescence intensity (MFI), and are from one lung preparation representative of two. The left panel shows an overlay histogram of primary antibody alone (dark histogram) and secondary antibody alone (open grey histogram). The right panel shows overlay histograms of titrated anti-LPA-1 receptor antibody (dark histogram) and secondary antibody alone (open grey histogram).

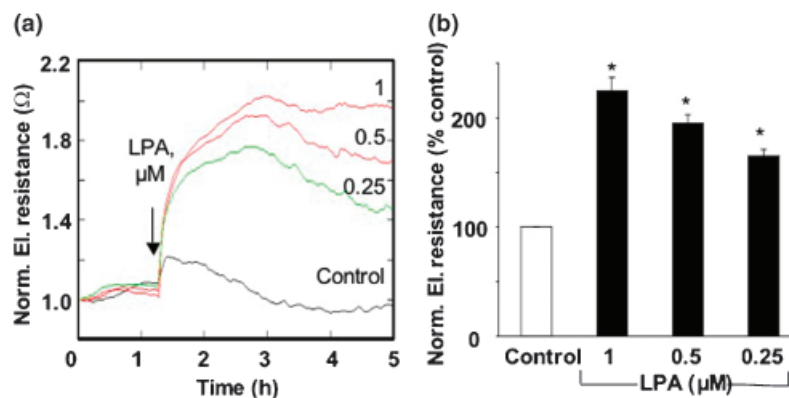


Fig. 7. Lysophosphatidic acid (LPA) promotes human airway epithelial cell barrier integrity. Normalized electrical resistance was measured using the electrical cell-substrate impedance system (ECIS, see Methods) after incubation with various concentrations of LPA as indicated. (a) Representative response to 0.25, 0.5 and 1 μ M LPA over time. (b) Average data from $n = 3$ experiments at 2 h. * $P < 0.05$ compared with control.

[18, 19], two cell types centrally involved in asthmatic inflammation. Third, we recently showed that LPA augmented the production of IL-13, a central cytokine in asthma, from sub-maximally stimulated T cells [45]. However, whether or not LPA was detectable in the lung in asthma or other inflammatory conditions had been unclear. Here we show that LPA levels are significantly increased above baseline values following segmental allergen challenge.

There are two possible sources of LPA accumulation in the lung during the late-phase allergic reaction. First, LPA could be generated by enzymatic breakdown of membrane phospholipids. Second, increased LPA in allergen-challenged lung segments could reflect the transudation of LPA bound to serum proteins due to increased capillary permeability (e.g. from histamine and other acute mediators). It is currently difficult to distinguish between these

two possibilities. Since serum proteins including albumin are thought to be a major component of BAL protein following allergen challenge [46], the modest correlation between LPA and total protein levels (Fig. 5c) suggests at least a component from transudated serum. However, this association was not perfect and some subjects demonstrated large increases in LPA with modest changes in BAL protein. Platelet activation is known to accompany lung allergen challenge [47] and also to generate LPA [7], providing one potential source of LPA following allergen challenge. Future studies will be needed to distinguish between these (and other) possibilities.

Total LPA increased ~3-fold following allergen challenge, although analysis of the fatty acid profile in a subset of subjects indicated that certain LPA molecular species were increased significantly more. Notable among these were docosahexaenoic (22:6)- and arachidonate (20:4) LPA, which increased ~22-fold and ~7-fold, respectively (Fig. 3). The enrichment of polyunsaturated species of LPA is notable for two reasons. First, the degree of polyunsaturation may affect how LPA interacts with its receptors [48]. Second, free docosahexaenoic acid can be converted to docosatrienes and the 17S series resolvins, which are currently thought to exert anti-inflammatory effects during the resolution of inflammation [49]. Whether LPA is a source of free fatty acids in the lung remains to be determined. Interestingly, LPA was consistently detectable in BAL fluids from saline-challenged control lung segments (average 482 pmol/mL, range from 189 to 1334 pmol/mL), consistent with a prior analysis demonstrating lysophospholipids in control lung [33]. Assuming a dilution factor of ~100 during BAL [50], this indicates that baseline values of LPA in the lung may be in the nanomolar range.

Although the concentration of LPA was significantly increased in the lung following allergen challenge, it did not correlate with the influx of total leucocytes or eosinophils measured 18 h after challenge. We can not rule out the possibility that LPA correlated with eosinophil numbers at earlier or later time-points after allergen challenge. Furthermore, it remains possible that LPA can activate eosinophils within the lung, as recently reported by Idzko et al. [19]. The recruitment of eosinophils to the allergic lung is a complex event driven by cytokines, adhesion molecules and chemokines [51, 52]: LPA does not appear to play a major role in this at least at this later time point. It is also possible that LPA levels could be increased in asthmatic subjects at baseline compared to controls, where it could contribute to the recruitment of eosinophils before allergen challenge. However, the lack of correlation between LPA levels and eosinophil numbers from saline-challenged control sites argues against this possibility. We did not measure airway obstruction in our subjects, and it remains possible that LPA levels could correlate with bronchospasm since it can augment airway smooth mus-

cle cell constriction. Potential associations between LPA and airway hyper-responsiveness will need to be addressed in future studies.

In contrast to eosinophils, the factors responsible for the recruitment of neutrophils to the lung during allergic inflammation are not as well understood. Allergen-dependent neutrophil recruitment in mice depends on antibodies and Fc- γ receptors (e.g. Fc γ RIII [53]): the role of this pathway in humans remains to be determined. In human allergen challenge experiments, endotoxin contamination may result in neutrophil recruitment, although even "endotoxin-free" allergen results in BAL neutrophilia [54]. Our data show that neither LPA nor IL-8 were associated with neutrophil recruitment following allergen challenge. Interestingly, Teran et al. also reported the lack of association between IL-8 levels and neutrophil recruitment during late-phase allergic reactions [55]. Although there was a correlation at earlier time-points in that report (4 h after challenge), neutrophil influx did not appear to be allergen-specific since it was also observed following saline challenge [55]. The potential role of other neutrophil chemoattractants will need to be addressed in subsequent studies.

We found that the levels of the chemokine MIP-3 α (CCL20) were increased following allergen challenge, and strongly correlated with lymphocyte numbers (Fig. 5). Lymphocytes are known to express the MIP-3 α receptor CCR6, which is an important regulator of lymphocyte trafficking [56]. Our data support the idea that the MIP-3 α -CCR6 pathway contributes to lymphocyte recruitment to the lung in allergic diseases. It will be interesting in future studies to determine if LPA levels correlate with markers of lymphocyte activation since LPA can augment T cell cytokine production *in vitro* [45]. MIP-3 α is also chemoattractant for dendritic cells [57], although we did not specifically enumerate these cells in our studies. Interestingly, we also observed significant correlations between MIP-3 α and the numbers of neutrophils and eosinophils in BAL. Because studies to-date have found that eosinophils and neutrophils do not express CCR6 or respond to MIP-3 α *in vitro* [58, 59], these correlations may reflect the production of MIP-3 α from granulocytes [60], or indirect effects of MIP-3 α on other cells. Nonetheless, our data provide one the first demonstration, to our knowledge, that MIP-3 α is increased in the allergic human airway, and support the idea that antagonizing the MIP-3 α -CCR6 pathway will attenuate lymphocyte recruitment to the lung.

The source of the constitutive LPA in the lung is presently not known, but the inverse correlation with BAL protein (Fig. 4d) suggested an association between LPA and lung barrier function in the absence of lung inflammation. This possibility was supported by *in vitro* studies showing that incubation with LPA significantly increased trans-epithelial resistance of the epithelial

monolayer (Fig. 7). Thus in addition to promoting the integrity of endothelial cells [22, 23], these data indicate that LPA also promotes airway epithelial barrier function. The barrier promoting properties of LPA may be overwhelmed in the presence of strong inflammation, as in the case of allergen challenge. These findings raise the possibility that LPA may instead play a role during resolution of inflammation and help restore barrier function once the inflammatory insult has subsided.

In summary, our studies provide some of the first evidence that LPA, a bioactive lysophospholipid, is increased in the lung during allergen-driven inflammation. LPA may interact with other mediators and promote cell activation or proliferation, but it does not appear to be a dominant chemoattractant for eosinophils, neutrophils or lymphocytes during late-phase allergic reactions. Given its effects as a growth factor on numerous cell types, repeated and prolonged exposure to LPA may promote airway remodelling. In contrast, LPA at baseline may have a barrier protective effect by promoting the integrity of the epithelial cell layer. It should be worthwhile in future studies to investigate the generation of different LPA molecular species and expression of LPA receptors at different stages of asthma.

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