Stimulated Bronchial Epithelial Cells Release Bioactive Lysophosphatidylcholine 16:0, 18:0, and 18:1

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Purpose: In human subjects and animal models with acute and chronic lung injury, the bioactive lysophosphatidylcholine (LPC) is elevated in lung lining fluids. The increased LPC can promote an inflammatory microenvironment resulting in lung injury. Furthermore, pathological lung conditions are associated with upregulated phospholipase A_2 (PLA_2), the predominant enzyme producing LPC in tissues by hydrolysis of phosphatidylcholine. However, the lung cell populations responsible for increases of LPC have yet to be systematically characterized. The goal was to investigate the LPC generation by bronchial epithelial cells in response to pathological mediators and determine the major LPC species produced. **Methods:** Primary human bronchial epithelial cells (NHBE) were challenged by vascular endothelial growth factor (VEGF) for 1 or 6 hours, and condition medium and cells collected for quantification of predominant LPC species by high performance liquid chromatography-tandem mass spectrometry (LC-MS-MS). The cells were analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for PLA_2. The direct effects of LPC in inducing inflammatory activities on NHBE were assessed by transepithelial resistance as well as expression of interleukin-8 (IL-8) and matrix metalloproteinase-1 (MMP-1). **Results:** VEGF stimulation of NHBE for 1 or 6 hours, significantly increased concentrations of LPC16:0, LPC18:0, and LPC18:1 in condition medium compared to control. The $sPLA_2$-selective inhibitor (oleyloxyethyl phosphorylcholine) inhibited the VEGF-induced release of LPC16:0 and LPC18:1 and PLA_2 activity. In contrast, NHBE stimulated with TNF did not induce LPC release. VEGF did not increase mRNA of PLA_2 subtypes $sPLA_2$-X, $sPLA_2$-XIIa, $cPLA_2$-IVa, and $iPLA_2$-VI. Exogenous LPC treatment increased expression of IL-8 and MMP-1, and reduced the transepithelial resistance in NHBE. **Conclusions:** Our findings indicate that VEGF-stimulated bronchial epithelial cells are a key source of extracellular LPCs, which can function as an autocrine mediator with potential to induce airway epithelial inflammatory injury.

Key Words: Bronchial epithelium; phospholipase A_2; lysophosphatidylcholine

INTRODUCTION

Lysophosphatidylcholine (LPC) is a pro-inflammatory and pro-atherogenic lipid mediator found at elevated levels in the broncho-alveolar lavage fluids (BALF) from lungs with acute and chronic inflammatory injury. For example, increased LPC levels are detected in BALF in allergic asthmatic subjects challenged with antigen[1] and in patients with late stage adult respiratory distress syndrome (ARDS).[2] In the *in vivo* guinea pig models of acute lung injury (ALI), increased levels of LPC are detected in BALF from lungs challenged with lipopolysaccharide[3] or H_2O_2.[4] Such pathological increases of LPC can significantly contribute to the inflammatory microenvironment in lungs. Abundant evidence indicates that LPC induces multiple pro-inflammatory activities, including promotion of cell growth,[5] migration,[6,7] secretion of chemokines and cytokines,[8,9] genera-

tion of reactive oxygen species,[10] and upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and selectins.[11] Furthermore, studies show that exogenous administration of LPC into lungs induces infiltration of eosinophils[12] and increased lung permeability.[13,14] However, the cellular populations responsible for LPC production have yet to be systematically characterized.

In tissues, LPC is generated predominantly by the enzyme

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• There are no financial or other issues that might lead to conflict of interest.

phospholipase A_2 (PLA$_2$), which hydrolyzes native and oxidized phosphatidylcholines at the *sn*-2 position, generating LPC following release of a free fatty acid.[15,16] PLA$_2$ is comprised of a superfamily of 15 distinct groups, each with numerous subtypes that varies with tissues.[15,16] Evidence indicates that pathological lung conditions such as pneumonia,[17] asthma,[1,18,19] experimental ALI,[20,21] can upregulate several PLA$_2$ isotypes in lung tissues, particularly airway epithelium and leukocytes. This suggests that these lung cell populations are likely major contributors to pathological increases in LPC content in the lung lining fluids.

Both acute and chronic lung diseases are associated with prototypic inflammatory mediators such as vascular endothelial growth factor (VEGF).[22-25] In this study, we investigated the hypothesis that VEGF stimulates bronchial epithelial cells to release major species of LPC. Additionally, we determined the pro-inflammatory potential of extracellular LPC on bronchial epithelial cells themselves.

MATERIALS AND METHODS

Materials

General reagents were obtained as follows: Bronchial Epithelial Cell Medium BulletKit (BEGM) (Lonza, Walkersville, MD, USA); Trizol, Hank's Balanced Salt Solution (HBSS), Protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), Red/Green BODIPY® PC-A$_2$ (Invitrogen Corp., Carlsbad, CA, USA); dimethylsulfoxide (DMSO), porcine pancreas PLA$_2$, lipopolysaccharide (*E. coli*) (Sigma-Aldrich, Inc.); Oleyloxyethyl phosphorylcholine (Enzo Life Sciences, Farmingdale, NY, USA); RQ1 RNase-free DNase, GoTaq® Green Master (Promega Corporation, Madison, WI, USA); Coomassie Plus Protein Assay (Pierce, Rockford, IL, USA); High Capacity cDNA Reverse Transcription Kit, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA); LPC16:0 (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine), LPC18:0 (1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine), LPC18:1 (1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine), LPC17:0 (1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine), and LPC19:0 (1-nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine) (Avanti Polar Lipids Inc., Alabaster, AL); human vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF) (Fisher BioServices, Pittsburgh, PA, USA).

Methods

Cell culture

Normal human bronchial epithelial cells (NHBE) (Lonza Walkersville, Inc., Walkersville, MD, USA) were obtained as second passage cells, with each batch derived from 3-5 donors. The NHBE were subcultured with BEGM according to manufacturer's instructions and used at population passage between 6 and 9. Two batches were used for the entire study, and spot checks of the cells' responses were made to evaluate for differences.

Treatment protocol

Human VEGF was dissolved in the sterile PBS to make a 5 μg/mL working stock solution, which was aliquotted and stored at -80°C. The NHBE, grown in 6-well culture dishes to confluence, were first equilibrated with HBSS for 1 hour, then treated with the appropriate final concentration of VEGF. At selected time-points, the condition medium was collected, centrifuged to remove cells, and stored in -80°C for subsequent analyses. The remaining cells were harvested by scraping and similarly stored for analyses. In a parallel set of studies, NHBE were treated with TNF, and similarly collected for analyses.

Purified LPC16:0 in chloroform was vacuum-dried, and was resuspended in 100% ethanol. The solution was re-evaporated and suspended in sterile HBSS (no albumin) to produce a working stock solution of 1 mM, which was stored at 4°C. For study, the LPC stock solution was diluted to the needed final concentration in HBSS, and added to confluent cultures of NHBE as described.

Quantification of lysophosphatidylcholine species

Glassware instead of plastic was used throughout sample handling to minimize analyte loss due to adsorption. Aliquots (0.5 mL) of collected samples were prepared for analysis by high performance liquid chromatography-tandem mass spectrometry (LC-MS-MS). The samples were spiked with LPC19:0 (140 ng/mL) as an internal standard and extracted twice using liquid/liquid extraction (chloroform/methanol, 2:1, v/v). The mixture was vortex-mixed for 5 minutes followed by centrifugation (5 minutes at $1,500 \times g$, 4°C), the organic layer was removed and saved, and the aqueous layer was extracted again. The organic layers were combined, evaporated to dryness under a stream of nitrogen, and the residue was reconstituted in 100 μL of methanol, of which 10 μL was injected into the LC-MS-MS system for analysis.

LC-MS-MS analysis was carried out using a Thermo (San Jose, CA, USA) TSQ Quantum triple quadrupole mass spectrometer equipped with a Waters (Milford, MA, USA) 2695 HPLC system and a Waters XTerra C_{18} column (2.1×100 mm, 3.5 μm). The solvent system consisted of a 15 mL linear gradient from 70%-100% methanol (containing 0.1% formic acid). The column was re-equilibrated with at least 10 column volumes between injections. LPCs were measured using positive electrospray mass spectrometry with collision-induced dissociation (CID) and selected reaction monitoring (SRM). The electrospray capillary temperature was 320°C, the spray voltage was 3,400 V, the tube lens offset was 100 V, and the sheath gas and auxiliary gas pressures were 39 psi and 20 psi, respectively. Argon at 1.5 Torr and 22 eV was used as the collision gas for CID. The SRM transitions for LPC16:0, 18:0, 18:1, and 19:0 were *m/z* 496 to *m/z* 184, *m/z* 524 to *m/z* 184, *m/z* 522 to *m/z* 184, and *m/z* 538 to *m/z* 184, respectively, and the dwell time was 0.2 sec/ion. The limit of detection of LPC was 10 pg (1 ng/mL; 10 μL injection) on-column based on a signal-to-noise ratio of 3. The limit of quantitation

was 25 pg (2.5 ng/mL; 10 μL injection) based on a signal-to-noise ratio of 10. The standard curves for LPCs in the condition medium samples over the concentration range of 2.5-500 ng/mL were linear with a coefficient of determination (R^2) >0.995. This assay showed excellent extraction efficiency, selectivity, sensitivity, precision, and accuracy.

RT-PCR

Total RNA from collected NHBE was isolated using Trizol according to manufacturer's instructions and was quantified by absorbance at 260 nm using the NanoDrop DNA/RNA/protein spectrophotometer (Thermo Fisher Scientific). One μg RNA was treated with RQ1 RNase-free DNase prior to reverse transcription reaction for cDNA synthesis using the high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Primers were designed to recognize human target genes and internal control genes by using Integrated DNA Technologies as follows:

a) sPLA$_2$-IIa (NM_000300): forward 5' ATCGCTGCTGTGT-CACTCAT 3', reverse 5' TTGCACAGGTGATTCTGCTC 3'

b) cPLA$_2$-IVa (NM_024420): forward 5' ACGTGATGTGCCT-GTGGTAGCC 3', reverse 5' GGTGGAGCCAGAAAGAC-CAGCA 3'

c) sPLA$_2$-V (NM_000929): forward 5' TTGGGCGCATGAC-CACTGCT 3', reverse 5' CCGGGCTCGCAGGTGACCA 3'

d) iPLA$_2$-VI (NM_003560): forward 5' CGTCTTCCATTAT-GCTGTCC 3', reverse 5' GGTCAGCCCTTGGTTATTCA 3'

e) sPLA$_2$-X (NM_003561): forward 5' CCGGCGAGGCCTC-CAGGATA 3', reverse 5' CGATGGGGGTTCGGGGACCA 3'

f) sPLA$_2$-XIIa (NM_030821): forward 5' TGTTTGGTGT-TCATCTTAACATTGG 3', reverse 5' CATCACAGTCAT-TCTTGCTTTT 3'

g) Interleukin-8 (IL-8, NM_000584): forward 5' CTCTTG-GCAGCCTTCCTGATT 3', reverse 5' TATGCACTGACATC-TAAGTTCTTTAGCA 3'

h) Matrix metalloproteinase-1 (MMP-1; NM_002421): forward 5' TGTGGACCATGCCATTGAGAA 3', reverse 5' TCT-GCTTGACCCTCAGAGACC 3'

i) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046): forward 5' ATGGCAAATTCCATGGCACC-GT 3', reverse 5' GCTCCTGGAAGATGGTGAT 3'.

For regular RT-PCR, the cDNA was amplified by using GoTaq® Green Master at the following step-wise conditions: 94°C×3 minutes; 35 cycles at 94°C×45 seconds; 60°C×45 seconds; 72°C×1 minute; then 72°C×15 minutes. PCR products were run in 1.5% agarose gel and visualized by staining with ethidium bromide and UV transillumination. For quantitative (q)RT-PCR, the cDNA was amplified with SYBR® Green PCR master mix kit using the BIORAD MyiQ single color real-time PCR detection system (Hercules, CA, USA) in a 96-well format. The amplification conditions were as follows: 50°C×2 minutes; 95°C×10 minutes; and 40 cycles at 95°C×15 seconds, 60°C×1 minute. Control reactions lacking template were run for each gene, and all samples were assayed in triplicate reactions.

Phospholipase A$_2$ activity

The condition medium from the experimental groups was measured for PLA$_2$ activity using a microplate assay based on the phosphatidylcholine substrate analog Red/Green BODIPY® PC-A$_2$, which produces a fluorescent product after PLA$_2$ cleavage. For the reaction, Red/Green BODIPY® PC-A$_2$ was added to 100 μL of condition medium at a final concentration of 1.7 μg/mL, and fluorescence measured at 515 nm at 10 minutes intervals for 2 hours in the plate reader (SpectraMax M5, Molecular Devices). An initial stock solution of 1 mg/mL of Red/Green BODIPY® PC-A$_2$ was made with DMSO, which was used for preparing the working solution with 50 mM Tris, 100 mM NaCl, 1 mM CaCl$_2$, pH 7.4.

Interleukin-8 protein quantification

The condition medium from the experimental groups was measured for IL-8 using an enzyme-linked immunoabsorbent assay (ELISA) kit (BioLegend, San Diego, CA, USA). IL-8 detection was based on a biotin-avidin-horseradish peroxidase sandwich technique in a 96-well plate which was coated with human IL-8 monoclonal antibody. Absorbance was read at 450 nm in the plate reader (SpectraMax M5, Molecular Devices).

Transepithelial electrical resistance

Real-time measurement of transepithelial electrical resistance was measured using an electrical cell-substrate impedence-sensing (ECIS) system (Applied Biophysics in Troy, New York, USA) as previously reported by us.[26,27] For study, NHBE were grown to confluence in fibronectin-coated 500 μL wells, each housing a gold-plated electrode in a 8-well ECIS array format. On the day of experiment, NHBE were washed in HBSS (absence of albumin), and equilibrated in HBSS within the ECIS incubator for 1 hour prior to stimulation by LPC, and data points were collected for up to 12 hours.

Statistical analysis

Statistical analysis was performed with GraphPad InStat (Version 3.10, 2009) by using student's Newman Keuls and One-way ANOVA with post test for multiple comparisons and the paired *t*-test (two-tailed) for pair comparison. A value of $P<0.05$ was considered significant whereas a value of $P<0.01$ was considered as highly significant.

RESULTS

Lysophosphatidylcholine release by normal human bronchial epithelial cells

We determined the effects of VEGF stimulation of NHBE on the release of LPC16:0, LPC18:0, and LPC18:1, three major LPC

species known to be biologically most active. A representative chromatograph shows the detected changes in the LPC profile from condition medium collected from VEGF-stimulated NHBE (Fig. 1). We observed that NHBE released basal amounts of LPC16:0, LPC18:0, and LPC18:1 to the medium, ranging from 2-14 ng/mL (Fig. 2A). Within 1 hour of stimulation by VEGF (10 nM), all three LPCs were significantly increased above basal levels, after which LPC16:0 and LPC18:1 further increased at 6 hours (Fig. 2A). At 6 hours of VEGF stimulation, the extracellular LPCs released were 2-3-fold above basal levels. The NHBE were also collected for measurement of cell-associated LPC. Results indicated that the cell-associated LPC16:0, LPC18:0, and LPC18:1 were not significantly changed after VEGF stimulation for 1 or 6 hours (Fig. 2B).

In contrast to VEGF, TNF (100 ng/mL) did not stimulate generation of LPC in NHBE (data not shown), suggesting that LPC

release was mediator-selective.

Effects of vascular endothelial growth factor on phospholipase A₂ activity

To determine whether the VEGF-induced LPC generation was attributed to secreted PLA_2, we quantified PLA_2 enzyme activity in condition medium from VEGF-treated or untreated NHBE using the phosphatidylcholine substrate analog. A selective inhibitor of $sPLA_2$, oleyloxyethyl phosphorylcholine (OP) was used to inhibit secreted PLA_2. Results indicated that VEGF (10 nM) stimulation of NHBE for 1 hour caused a moderate increase in PLA_2 activity which was inhibited by OP (20 μM) (Fig. 3). The findings indicate that i) VEGF stimulation increased PLA_2 activity in the condition medium, and ii) OP inhibited VEGF-stimulated PLA_2 activity in condition medium.

We next determined whether the VEGF-induced increase in
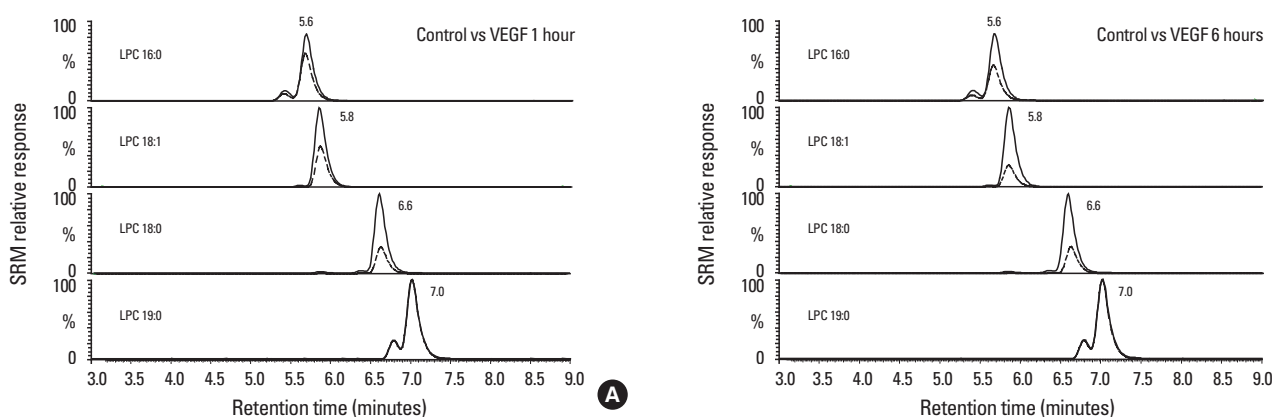

Fig. 1. Representative positive ion electrospray LC-MS-MS SRM chromatograms showing the detection of LPC species from condition medium collected from NHBE stimulated with (A) 10 nM VEGF for 1 hour or (B) 10 nM VEGF for 6 hours. SRM transitions: LPC 16:0 m/z 496→184 (retention time 5.6 minutes); LPC 18:0 m/z 524→184 (retention time 6.6 minutes); LPC 18:1 m/z 522→184 (retention time 5.8 minutes); LPC 19:0 m/z 538→184 (retention times 7.0 minutes). The solid line represents cells treated with VEGF, and the dashed line represents the control without VEGF.
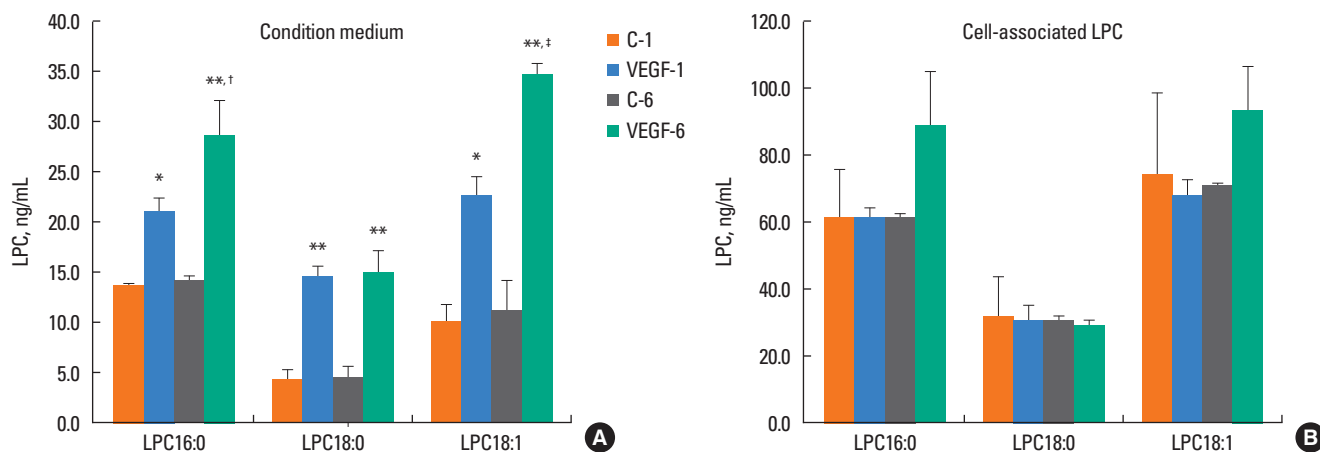VEGF, vascular endothelial growth factor.

Fig. 2. Summary graph showing the effects of VEGF stimulation on generation of LPC16:0, LPC18:0, and LPC18:1. NHBE were treated with 10 nM VEGF for 1 (VEGF-1) or 6 hours (VEGF-6) or with control buffer (C-1 and C-6, respectively); (A) the condition medium and (B) cells were collected for analysis by LC-MS-MS (Materials and Methods). *$P<0.05$ and **$P<0.001$ compared to control; †$P<0.01$ and ‡$P<0.001$, compared to 1 hour VEGF; n=3-4.

PLA$_2$ activity in the condition medium was attributed to upregulation of PLA$_2$ mRNA. Guided by reports from the literature, several major human PLA$_2$ subtypes were selected for an initial evaluation of their constitutive expression by regular RT-PCR, specifically, secretory subtypes (sPLA$_2$-IIa, sPLA$_2$-V, sPLA$_2$-X, and sPLA$_2$-XIIa), calcium-dependent cytosolic subtype (cPLA$_2$-IVa), and calcium-independent subtype (iPLA$_2$-VI). The initial results indicated that NHBE expressed constitutive robust levels of four subtypes sPLA$_2$-X, sPLA$_2$-XIIa, cPLA$_2$-IVa, and iPLA$_2$-VI, but barely detectable levels of either sPLA$_2$-IIa or sPLA$_2$-V (Fig. 4A). We next determined the effects of VEGF stimulation on the four PLA$_2$ subtypes by qRT-PCR analysis of the NHBE stimulated with VEGF (10 nM) for 1 or 6 hours. Results indicated that the mRNA of sPLA$_2$ subtypes, cPLA$_2$-IVa and iPLA$_2$-VI, were not significantly changed from baseline (Fig. 4B). These results indicate that the VEGF-stimulated LPC production was likely not attributed to increased *de novo* protein synthesis of these PLA$_2$s.

Effects of secretory phospholipase A$_2$ inhibitor on lysophosphatidylcholine generation

Since our results showed that the sPLA$_2$ inhibitor, OP, abrogated the VEGF-stimulated PLA$_2$ activity, we determined whether OP also inhibits the VEGF-stimulated LPC release. NHBE were pretreated with OP (20 μM for 30 minutes), then stimulated with VEGF (10 nM for 1 hour), and the condition medium collected for LPC determination by LC-MS-MS. The results indicated that OP abrogated the VEGF-stimulated levels of LPC16:0 and LPC18:1, but was not effective for LPC18:0 (Fig. 5). The findings suggest that the VEGF-induced increase of LPC16:0 and LPC18:1 (but not LPC18:0) was mostly attributed to secreted sPLA$_2$ in the condition medium.
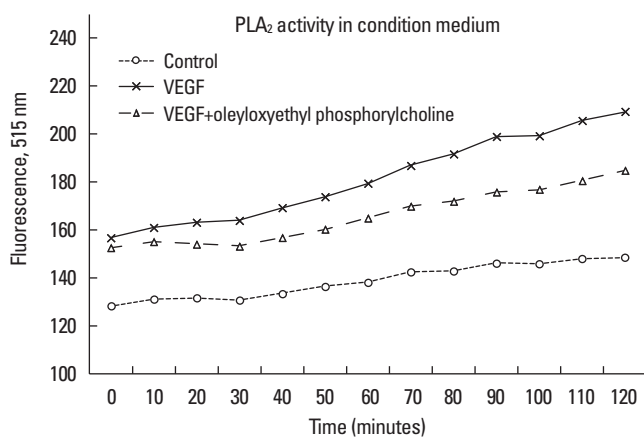
Effects of exogenous lysophosphatidylcholine on inflammatory injury

We observed that VEGF significantly increased the content of LPC16:0, LPC18:0, and LPC18:1 in the condition medium. This suggests that bronchial epithelium not only is a significant contributor to extracellular LPC in lungs, but also the bronchial epithelium itself is exposed to elevated levels of LPC. Therefore, we investigated whether the NHBE were susceptible to LPC's inflammatory activities.

The *in vivo* concentration of LPC in the lung lining fluids is not known, but our previous work[26,28] and work reported by others[29-31] indicate that an LPC concentration range of 10-100 μM is effective on a variety of cell types in inducing inflammatory activity. We tested the effects of LPC16:0 at comparable concentrations in inducing expression of inflammatory molecules and in impairing the bronchial epithelial barrier. For study, NHBE were grown to confluence in 6-well culture dishes, treated with 25 μM LPC, and the condition medium and cells collected for assessment of inflammatory molecules. The results indicated that stimulation with LPC for 5 hours, but not 1 hour, significantly upregulated IL-8 mRNA (Fig. 6A) and protein (Fig. 6B). QRT-PCR of another pro-inflammatory gene, MMP-1, indicated that stimulation with LPC for 5 hours also increased MMP-1 mRNA (Fig. 7). The effects of LPC on epithelial barrier
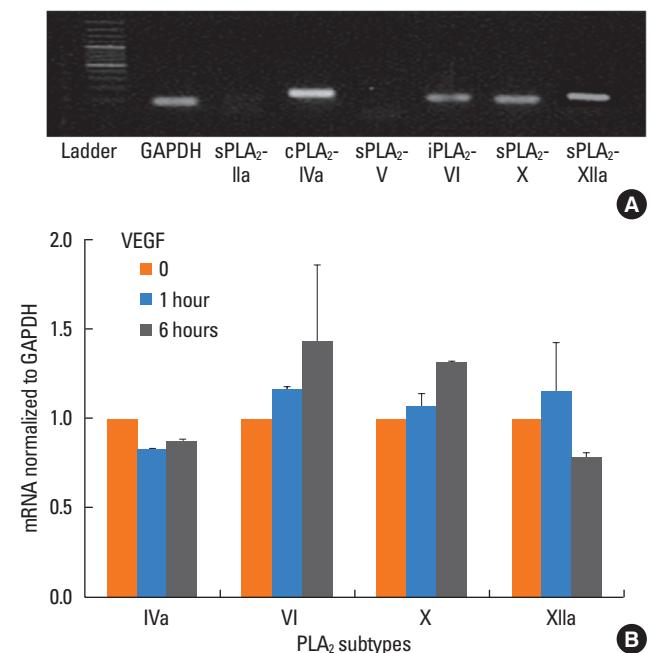
Fig. 4. PLA$_2$ expression by NHBE. (A) Shown is a representative gel of regular RT-PCR analysis of constitutive expression of major PLA$_2$ subtypes in NHBE and GAPDH used as an internal control. Major sPLA$_2$ subtypes are: sPLA$_2$-IIa, sPLA$_2$-V, sPLA$_2$-X and sPLA$_2$-XIIa; other subtypes are: cPLA$_2$-IVa and iPLA$_2$-VI; n=3. (B) qRT-PCR analysis of PLA$_2$ mRNA in response to VEGF treatment. NHBE were treated with VEGF (10 nM for 1 or 6 hours), and collected for quantification of sPLA$_2$-X, sPLA$_2$-XIIa, cPLA$_2$-IVa, and iPLA$_2$-VI. Results reported as relative copy number normalized to GAPDH and shown as average ± SE; n=2.

Fig. 3. Increased PLA$_2$ activity in condition medium by VEGF. The PLA$_2$ activity in the condition medium from VEGF-stimulated or untreated (control) NHBE was determined using a phosphatidylcholine substrate analog (Red/Green BODIPY PC-A$_2$) (Materials and Methods). Activity was measured at 10 minutes intervals for 120 minutes in the presence or absence of a selective inhibitor of sPLA$_2$, oleyloxyethyl phosphorylcholine (oleyloxyethyl phosphorylcholine; 20 μM); n=2.
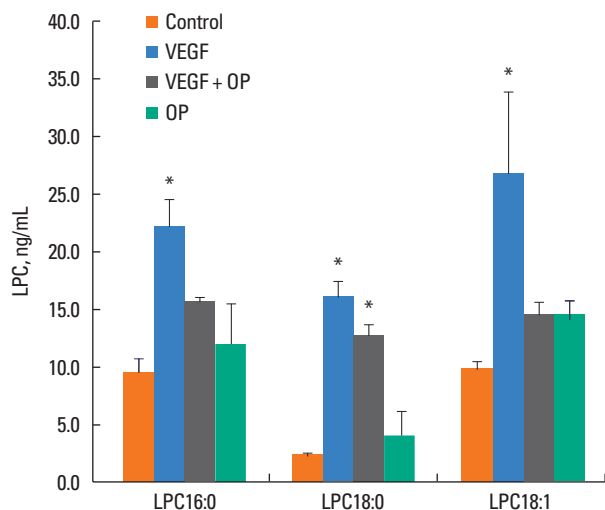
Fig. 5. Effects of sPLA₂ inhibitor on LPC generation. The effect of the sPLA₂-selective inhibitor, oleyloxyethyl phosphorylcholine (Op), on the VEGF-induced release of LPC16:0, LPC18:0, and LPC18:1 into the condition medium was determined. OP (20 µM) was added to the medium as a pretreatment of NHBE for 30 minutes, followed by treatment with VEGF (10 nM for 1 hour) or buffer control, and the condition medium was collected for LPC determination by LC-MS-MS as described. * $P < 0.05$, compared to control; n=3-7.
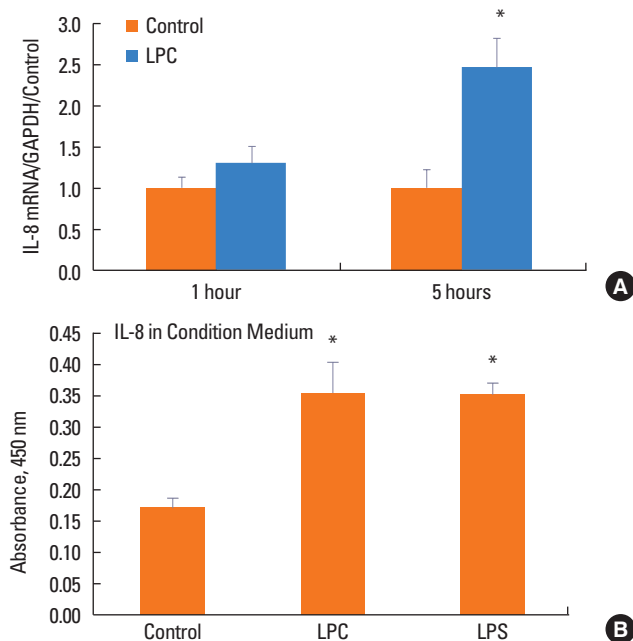

Fig. 6. Upregulation of IL-8 by exogenous LPC. (A) NHBE were treated with LPC16:0 (25 µM) for 1 or 5 hours, and cells collected for qRT-PCR analysis of IL-8 mRNA; the graph shows relative copy number of IL-8 mRNA normalized to GAPDH as average ± SE. (B) NHBE were treated with LPC16:0 (25 µM) for 4 hours, and the condition medium collected for secreted IL-8 protein; results reported as average relative absorbance (450 nm); LPS treatment (10 µg/mL) for 4 hours was used as positive control. N=4. *$P < 0.05$ compared to control.
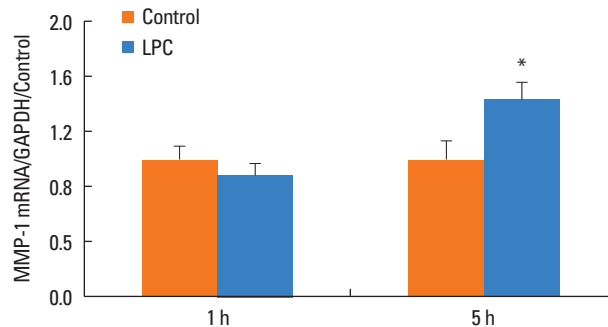

Fig. 7. Upregulation of MMP-1 by exogenous LPC. NHBE cells were treated with LPC16:0 (25 µM) for 1 or 5 hours, and cells collected for qRT-PCR analysis for MMP-1 mRNA; graph shows the relative copy number of MMP-1 normalized to GAPDH as average ± SE. *$P < 0.05$ compared to control; n=4-8.
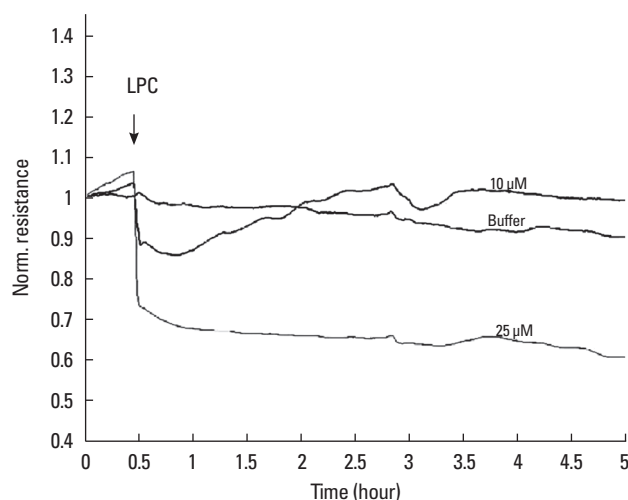

Fig. 8. Decreased transepithelial electrical resistance by LPC. Confluent NHBE were grown on ECIS electrodes, and real-time resistance change in response to LPC16:0 stimulation was measured for up to 5 hours; shown is representative resistance change relative to baseline (from 6 separate determinations); arrow indicates time of LPC addition.

function were assessed by the transepithelial resistance across monolayers of NHBE. Treatment with LPC (10 and 25 µM) caused a rapid decrease in resistance (Fig. 8). At the lower LPC concentration, the decreased resistance returned to near baseline by ~2 hours; whereas at the higher concentration, resistance decreased maximally to ~35% below baseline and did not show reversal for up to 12 hours. Overall, the findings indicate that bronchial epithelial cells were susceptible to the pro-inflammatory effects of LPC.

DISCUSSION

The central finding from this study is that VEGF stimulated a 2-4-fold release of LPC16:0, LPC18:0, and LPC18:1 by bronchial epithelial cells. These LPC species are the most abundant and also appear to be most significant in exerting inflammatory activities when compared with several other LPCs. In one report, LPC16:0, LPC18:0, and LPC18:1 induce neutrophil priming, but

LPC12:0, LPC14:0, and LPC17:0 were ineffective.[32] Further, LPC16:0 and LPC18:0 are observed to be more potent than LPC18:1 in stimulating eosinophil adhesion.[33] Our results indicate that stimulated bronchial epithelial cells release these bioactive LPCs which can contribute to the promotion of an inflammatory lung microenvironment. The significance of the finding is underscored by reports that the BALF from subjects with asthma and ARDS, as well as experimental animal models with acute and chronic lung injury contain elevated levels of LPC.[1-4]

The findings indicate that the VEGF-stimulated increase in extracellular LPC was most likely attributed to secreted extracellular PLA_2 and not intracellular PLA_2. Three observations are consistent with this conclusion. First, VEGF did not increase the cell-associated LPC content. We reason that if VEGF stimulated intracellular production of LPC, the cell-associated LPC ought to be increased. Second, VEGF stimulated an increase in PLA_2 activity in the condition medium. Third, VEGF did not increase mRNA of any of the PLA_2 subtypes, therefore, the increase in extracellular PLA_2 activity must be attributed to secreted pre-formed PLA_2. Our observation that only VEGF, and not TNF, induced LPC release was surprising since both are recognized mediators frequently found in lungs with acute and chronic injury. This selectivity by mediators in inducing LPC generation will be an important direction for future investigation.

The $sPLA_2$-selective inhibitor, OP, inhibited VEGF-induced $sPLA_2$ activity as well as the generation of LPC16:0 and LPC18:1, but not LPC18:0. This finding provides a causal link between the extracellular LPC and extracellular PLA_2, and indicates that $sPLA_2$ is likely a predominant subtype for LPC generation (at least for LPC16:0 and LPC18:1) by NHBE. The finding that OP inhibited ~40% of the VEGF-stimulate PLA_2 activity in the condition medium may be related to multiple factors. It appears that the efficacy of the inhibitor is likely dependent on the cell type or tissue. This is in agreement with reports by others who have used OP at a range of 1-100 μM, with varying degrees of inhibition depending on the experimental model.[34-36] Further, although the reported IC50 data from the manufacturer indicates that OP prefers $sPLA_2$ over $cPLA_2$, the extent of inhibition can vary between subtypes among $sPLA_2$ family members and even between different species of tissue origin (e.g., IC50 for purified porcine pancreatic $sPLA_2$ is 6.2 μM, but for human pancreatic $sPLA_2$ is 13.7 μM). In our optimization studies, OP inhibited purified porcine pancreatic and bee venom $sPLA_2$ with highly distinct kinetics. There are 9 members belonging to the human $sPLA_2$ subgroup, and it is not known which ones are actually secreted by the bronchial epithelium. We suspect that if VEGF stimulated secretion of multiple $sPLA_2$ subtypes, OP may only be partial effective in blocking the PLA_2 activity and as well the LPC generation.

Our findings indicate that the airway epithelium can be a source for LPC release to the lung lining fluids. This suggests that in the *in vivo* lung, the airway epithelium can be exposed to elevated levels of LPC. Therefore, we investigated whether NHBE are susceptible to inflammatory injury mediated by exogenous LPC. LPC is known to activate several transcription factors, including nuclear factor-kappa B (NFκB), which are linked to inflammatory genes.[37-40] We found that LPC upregulated pro-inflammatory genes, IL-8 and MMP-1, further strengthening our hypothesis that increased extracellular LPC is injurious to the bronchial epithelium.

LPC also decreased the transepithelial resistance, indicating an acute impairment of the epithelial barrier function. Our results are consistent with reports that exogenous LPC increases alveolar and airway epithelium permeability in isolated perfused lungs.[13,14,41] The extent of the LPC-induced resistance drop is comparable to that of alcohol (25%-40%) treatment of bronchial epithelial cells, which is associated with redistribution of tight junctional proteins.[42] The upstream signals that regulate the junctional redistribution are not well understood. We have found that the LPC-induced endothelial barrier dysfunction is likely acting through the lysophospholipid receptor, GPR4,[26] which activates protein kinase C and Rho GTPases.[27] The role of GPR4 in the LPC-induced inflammatory activities of the bronchial epithelium will be an important direction for future investigation.

In conclusion, findings from this study provide clear evidence that bronchial epithelial cells (NHBE) released bioactive LPC16:0, LPC18:0, and LPC18:1 in response to VEGF. The increased release of LPCs was likely attributed to increased extracellular PLA_2 activity. Moreover, NHBE were susceptible to the inflammatory activity of exogenous LPC, indicative of an autocrine function. Altogether, our study shows that bronchial epithelial cells produce extracellular LPC, which has potential to induce inflammatory injury of airway epithelium.

ACKNOWLEDGMENTS

REFERENCES

1. Chilton FH, Averill FJ, Hubbard WC, Fonteh AN, Triggiani M, Liu MC. Antigen-induced generation of lyso-phospholipids in human airways. J Exp Med 1996;183:2235-45.
2. Nakos G, Kitsiouli EI, Tsangaris I, Lekka ME. Bronchoalveolar lavage fluid characteristics of early intermediate and late phases of ARDS. Alterations in leukocytes, proteins, PAF and surfactant components. Intensive Care Med 1998;24:296-303.
3. Arbibe L, Koumanov K, Vial D, Rougeot C, Faure G, Havet N, Longacre S, Vargaftig BB, Béréziat G, Voelker DR, Wolf C, Touqui L. Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a di-

rect surfactant protein A-phospholipase A2 protein interaction. J Clin Invest 1998;102:1152-60.

4. Rice KL, Duane PG, Archer SL, Gilboe DP, Niewoehner DE. H2O2 injury causes Ca(2+)-dependent and -independent hydrolysis of phosphatidylcholine in alveolar epithelial cells. Am J Physiol 1992; 263:L430-8.

5. Schaefer CA, Kuhlmann CR, Gast C, Weiterer S, Li F, Most AK, Neumann T, Backenkohler U, Tillmanns H, Waldecker B, Wiecha J, Erdogan A. Statins prevent oxidized low-density lipoprotein- and lysophosphatidylcholine-induced proliferation of human endothelial cells. Vascul Pharmacol 2004;41:67-73.

6. Radu CG, Yang LV, Riedinger M, Au M, Witte ON. T cell chemotaxis to lysophosphatidylcholine through the G2A receptor. Proc Natl Acad Sci U S A 2004;101:245-50.

7. Kohno M, Yokokawa K, Yasunari K, Minami M, Kano H, Hanehira T, Yoshikawa J. Induction by lysophosphatidylcholine, a major phospholipid component of atherogenic lipoproteins, of human coronary artery smooth muscle cell migration. Circulation 1998;98:353-9.

8. Liu-Wu Y, Hurt-Camejo E, Wiklund O. Lysophosphatidylcholine induces the production of IL-1beta by human monocytes. Atherosclerosis 1998;137:351-7.

9. Spangelo BL, Jarvis WD. Lysophosphatidylcholine stimulates interleukin-6 release from rat anterior pituitary cells in vitro. Endocrinology 1996;137:4419-26.

10. Takeshita S, Inoue N, Gao D, Rikitake Y, Kawashima S, Tawa R, Sakurai H, Yokoyama M. Lysophosphatidylcholine enhances superoxide anions production via endothelial NADH/NADPH oxidase. J Atheroscler Thromb 2000;7:238-46.

11. Zou Y, Kim CH, Chung JH, Kim JY, Chung SW, Kim MK, Im DS, Lee J, Yu BP, Chung HY. Upregulation of endothelial adhesion molecules by lysophosphatidylcholine. Involvement of G protein-coupled receptor GPR4. FEBS J 2007;274:2573-84.

12. Nishiyama O, Kume H, Kondo M, Ito Y, Ito M, Yamaki K. Role of lysophosphatidylcholine in eosinophil infiltration and resistance in airways. Clin Exp Pharmacol Physiol 2004;31:179-84.

13. Niewoehner DE, Rice K, Sinha AA, Wangensteen D. Injurious effects of lysophosphatidylcholine on barrier properties of alveolar epithelium. J Appl Physiol 1987;63:1979-86.

14. Lindahl M, Hede AR, Tagesson C. Lysophosphatidylcholine increases airway and capillary permeability in the isolated perfused rat lung. Exp Lung Res 1986;11:1-12.

15. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 2009;50 Suppl:S237-42.

16. Burke JE, Dennis EA. Phospholipase A2 biochemistry. Cardiovasc Drugs Ther 2009;23:49-59.

17. Masuda S, Murakami M, Mitsuishi M, Komiyama K, Ishikawa Y, Ishii T, Kudo I. Expression of secretory phospholipase A2 enzymes in lungs of humans with pneumonia and their potential prostaglandin-synthetic function in human lung-derived cells. Biochem J 2005; 387:27-38.

18. Bowton DL, Seeds MC, Fasano MB, Goldsmith B, Bass DA. Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. Am J Respir Crit Care Med 1997;155:421-5.

19. Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson WR Jr. Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. Am J Respir Crit Care Med 2007; 176:1072-8.

20. Nakano T, Arita H. Enhanced expression of group II phospholipase

A2 gene in the tissues of endotoxin shock rats and its suppression by glucocorticoid. FEBS Lett 1990;273:23-6.

21. Ljungman AG, Tagesson C, Lindahl M. Endotoxin stimulates the expression of group II PLA2 in rat lung in vivo and in isolated perfused lungs. Am J Physiol 1996;270:L752-60.

22. Lopez-Guisa JM, Powers C, File D, Cochrane E, Jimenez N, Debley JS. Airway epithelial cells from asthmatic children differentially express proremodeling factors. J Allergy Clin Immunol 2012;129:990-7.e6.

23. Braber S, Henricks PA, Nijkamp FP, Kraneveld AD, Folkerts G. Inflammatory changes in the airways of mice caused by cigarette smoke exposure are only partially reversed after smoking cessation. Respir Res 2010;11:99.

24. Vasakova M, Sterclova M, Kolesar L, Slavcev A, Pohunek P, Sulc J, Skibova J, Striz I. Bronchoalveolar lavage fluid cellular characteristics, functional parameters and cytokine and chemokine levels in interstitial lung diseases. Scand J Immunol 2009;69:268-74.

25. Zhang J, Zheng L, Bai M. Dynamic expression of tumor necrosis factor-alpha and vascular endothelial growth factor in rat model of pulmonary emphysema induced by smoke exposure. J Huazhong Univ Sci Technolog Med Sci 2007;27:505-7.

26. Qiao J, Huang F, Naikawadi RP, Kim KS, Said T, Lum H. Lysophosphatidylcholine impairs endothelial barrier function through the G protein-coupled receptor GPR4. Am J Physiol Lung Cell Mol Physiol 2006;291:L91-101.

27. Huang F, Subbaiah PV, Holian O, Zhang J, Johnson A, Gertzberg N, Lum H. Lysophosphatidylcholine increases endothelial permeability: role of PKCalpha and RhoA cross talk. Am J Physiol Lung Cell Mol Physiol 2005;289:L176-85.

28. Huang F, Mehta D, Predescu S, Kim KS, Lum H. A novel lysophospholipid- and pH-sensitive receptor, GPR4, in brain endothelial cells regulates monocyte transmigration. Endothelium 2007;14:25-34.

29. Aiyar N, Disa J, Ao Z, Ju H, Nerurkar S, Willette RN, Macphee CH, Johns DG, Douglas SA. Lysophosphatidylcholine induces inflammatory activation of human coronary artery smooth muscle cells. Mol Cell Biochem 2007;295:113-20.

30. Han KH, Hong KH, Ko J, Rhee KS, Hong MK, Kim JJ, Kim YH, Park SJ. Lysophosphatidylcholine up-regulates CXCR4 chemokine receptor expression in human CD4 T cells. J Leukoc Biol 2004;76:195-202.

31. Murugesan G, Sandhya Rani MR, Gerber CE, Mukhopadhyay C, Ransohoff RM, Chisolm GM, Kottke-Marchant K. Lysophosphatidylcholine regulates human microvascular endothelial cell expression of chemokines. J Mol Cell Cardiol 2003;35:1375-84.

32. Silliman CC, Elzi DJ, Ambruso DR, Musters RJ, Hamiel C, Harbeck RJ, Paterson AJ, Bjornsen AJ, Wyman TH, Kelher M, England KM, McLaughlin-Malaxecheberria N, Barnett CC, Aiboshi J, Bannerjee A. Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium. J Leukoc Biol 2003;73:511-24.

33. Zhu X, Learoyd J, Butt S, Zhu L, Usatyuk PV, Natarajan V, Munoz NM, Leff AR. Regulation of eosinophil adhesion by lysophosphatidylcholine via a non-store-operated Ca2+ channel. Am J Respir Cell Mol Biol 2007;36:585-93.

34. Jino H, Kurahashi K, Usui H, Nakata Y, Shimizu Y. Possible involvement of endothelial leukotrienes in acetylcholine-induced contraction in rabbit coronary artery. Life Sci 1996;59:961-7.

35. Sim SS, Choi JC, Min DS, Rhie DJ, Yoon SH, Hahn SJ, Kim CJ, Kim MS, Jo YH. The involvement of phospholipase A(2) in ethanol-in-

duced gastric muscle contraction. Eur J Pharmacol 2001;413:281-5.

36. Daray FM, Colombo JR, Kibrik JR, Errasti AE, Pelorosso FG, Nowak W, Cracowski JL, Rothlin RP. Involvement of endothelial thromboxane A2 in the vasoconstrictor response induced by 15-E2t-isoprostane in isolated human umbilical vein. Naunyn Schmiedebergs Arch Pharmacol 2006;373:367-75.

37. Xing F, Liu J, Mo Y, Liu Z, Qin Q, Wang J, Fan Z, Long Y, Liu N, Zhao K, Jiang Y. Lysophosphatidylcholine up-regulates human endothelial nitric oxide synthase gene transactivity by c-Jun N-terminal kinase signalling pathway. J Cell Mol Med 2009;13:1136-48.

38. Masamune A, Sakai Y, Yoshida M, Satoh A, Satoh K, Shimosegawa T. Lysophosphatidylcholine activates transcription factor NF-kappaB and AP-1 in AR42J cells. Dig Dis Sci 2001;46:1871-81.

39. Ueno Y, Kume N, Miyamoto S, Morimoto M, Kataoka H, Ochi H, Nishi E, Moriwaki H, Minami M, Hashimoto N, Kita T. Lysophosphatidylcholine phosphorylates CREB and activates the jun2TRE site of c-jun promoter in vascular endothelial cells. FEBS Lett 1999; 457:241-5.

40. Fang X, Gibson S, Flowers M, Furui T, Bast RC Jr, Mills GB. Lysophosphatidylcholine stimulates activator protein 1 and the c-Jun N-terminal kinase activity. J Biol Chem 1997;272:13683-9.

41. Butler BD, Davies I, Drake RE. Effect of lysophosphatidylcholine on the filtration coefficient in intact dog lungs. Am J Physiol 1989; 257:H1466-70.

42. Simet SM, Wyatt TA, DeVasure J, Yanov D, Allen-Gipson D, Sisson JH. Alcohol increases the permeability of airway epithelial tight junctions in Beas-2B and NHBE cells. Alcohol Clin Exp Res 2012; 36:432-42.