Involvement of PLD2 in LPA - Induced Transactivation of Platelet-derived growth factor Receptor-β in Human Bronchial Epithelial Cells

Lixin Wang a, Rhett Cummings a, Yutong Zhao a, Andrius Kazlauskas b, James Sham a, Andrew Morris c, Steve Georas a, David N. Brindley d, and Viswanathan Natarajan a *

a Department of Medicine, Division of Pulmonary and Critical Care, Johns Hopkins University, Baltimore, MD 21224, b The Schepens Eye Research Institute, Harvard Medical School, Boston, MA 02114, c Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599 and d Department of Biochemistry, Signal Transduction Research Group, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada.

* Address Correspondence to:
Dr. V. Natarajan
Johns Hopkins University School of Medicine
Mason F. Lord Building, Room 675
5200 Eastern Evenue
Baltimore, MD 21224
Tel: 410-550-7748; Fax: 410-550-8571
e-mail: vnataraj@jhmi.edu

Short Title: Phospholipase D2 in PDGF-Rβ Transactivation by LPA

Key Words: Lysophosphatidic acid; Transactivation; Phospholipase D2; PDGF-Rβ; ERK1/2

1 The abbreviations used are: ERK, extracellular-signal regulated kinase; GPRC, G protein-coupled receptor; LPA, lysophosphatidate; LPA-1 to 3, LPA receptor; MAPK, mitogen activated protein kinase; PA, phosphatidate; PDGF-R, platelet-derived growth factor receptor; p.f.u., plaque forming units; PI 3-K, phosphatidylinositol 3-kinase; PLD, phospholipase D; PBT, phosphatidylbutanol; PTx, pertussis toxin; S1P, sphingosine-1-phosphate; TPA, 12-O-tetradecanoyl phorbol 13-acetate

Copyright 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Abstract

Lysophosphatidate (LPA), a bioactive lipid signaling molecule, mediates multiple cellular responses via heterotrimeric G-protein coupled LPA-1, LPA-2 and LPA-3 receptors (LPA-Rs). Many G-protein coupled receptors stimulate ERK following tyrosine phosphorylation of growth factor receptors; however, the mechanism(s) of transactivation of receptor tyrosine kinases are not well defined. Here, we provide evidence for the involvement of phospholipase D (PLD) in LPA–mediated transactivation of platelet-derived growth factor receptor-β (PDGF-Rβ). In primary cultures of human bronchial epithelial cells (HBEpCs), LPA in a dose-dependent manner stimulated tyrosine phosphorylation of PDGF-Rβ and threonine/tyrosine phosphorylation of ERK1/2. The LPA-mediated activation of ERK and tyrosine phosphorylation of PDGF-Rβ was attenuated by tyrphostin AG-1296, an inhibitor of PDGF-R kinase, suggesting transactivation of PDGF-R by LPA. Furthermore, LPA-, but not PDGF-BB-, induced tyrosine phosphorylation of PDGF-Rβ was partially blocked by pertussis toxin indicating coupling of LPA receptor(s) to Gi. Exposure of HBEpCs to LPA activated PLD. Butan-1-ol, which acts as an acceptor of phosphatidate generated by the PLD pathway by forming phosphatidylbutanol through the transphosphatidylation reaction, significantly blocked LPA-mediated transactivation of PDGF-Rβ. This effect was not seen with butan-3-ol, suggesting PLD involvement. The role of PLD1 and PLD2 in the PDGF-Rβ transactivation by LPA was investigated by infection of cells with adenoviral constructs of wild type and catalytically inactive mutants of PLD1 and PLD2. LPA activated both PLD1 and PLD2 in HBEpCs; however, infection of cells with cDNA for wild type PLD2, but not PLD1, increased the tyrosine phosphorylation of PDGF-Rβ in response to LPA.
Also, the LPA-mediated tyrosine phosphorylation of PDGF-Rβ was attenuated by the catalytically inactive mutant mPLD2-K758R while the inactive mutant hPLD1-K898R was ineffective. Infection of HBEpCs with adenoviral constructs of wild type hPLD1, mPLD2 and the catalytically inactive mutants, hPLD1-K898R and mPLD2-K758R resulted in association of PLD2 wild type and inactive mutant proteins with the PDGF-Rβ compared to PLD1. These results show for the first time that transactivation of PDGF-Rβ by LPA in human bronchial epithelial cells, is regulated by PLD2.
Introduction

Lysophosphatidate (LPA)\(^1\), is a potent bioactive lipid that is implicated in cell proliferation, differentiation, suppression of apoptosis, tumor metastasis and cytoskeletal reorganization (1-5). In plasma, LPA is present at < 0.2 \(\mu\)M, but activated platelets produce high amounts of LPA which can reach 1-10 \(\mu\)M, making it the most abundant mitogen / survival factor present in serum (6). Mammalian cells contain small quantities of intracellular LPA; however, many biological actions of LPA are mediated by specific G-protein coupled receptors (GPCRs), LPA-1, LPA-2 and LPA-3 (formerly called EDG 2, 4 and 7). LPA, akin to sphingosine-1-phosphate (S1P), binds with high affinity to its receptors that are coupled to multiple types of heterotrimeric G proteins (G\(_i\), G\(_q\) and G\(_{12/13}\)). Ligation of LPA to LPA-Rs leads to a myriad of signal transduction pathways including release of [Ca\(^{2+}\)]\(_i\), activation of protein kinase C, phosphatidylinositol-3-kinase (PI-3-K), mitogen activated protein kinases (ERK1 and ERK2), tyrosine kinases and phospholipases (7). LPA induced release of [Ca\(^{2+}\)]\(_i\) and decreased cAMP are pertussis toxin (PTx)-sensitive suggesting involvement of G\(_i\) (8). LPA-mediated activation of ERK1/2 requires p21 Ras (9) and is dependent on PI 3-K signaling through G\(_i\) (10). Furthermore, LPA activated the non-receptor tyrosine kinase, Src, and Pyk2 (11, 12). LPA-induced Rho activation through G\(_{12/13}\) results in stress fibers, assembly of focal adhesion and NF-κB-dependent gene transcription (13).

LPA activates PLD in various mammalian cells, including fibroblasts, smooth muscle cells and prostate cancer cells (14-16). PLD isoenzymes, PLD1 and PLD2 hydrolyze phosphatidylcholine to generate phosphatidate (PA) and they are activated by hormones,
growth factors, neurotransmitters, cytokines and reactive oxygen species (17). The
second messenger functions of PA are mediated directly or following its conversion to
either diacylglycerol or LPA by the action of PA phosphohydrolase or phospholipase
A1/A2, respectively. PA derived through PLD regulates protein tyrosine phosphatases,
ERK1/2 phosphorylation, phosphatidylinositol 4-kinase, PI-3-kinase,
phosphatidylinositol 4-phosphate 5-kinase, phagocytic NADPH oxidase, actin
polymerization, coatamer assembly, vesicle trafficking and cytokine secretion (18-21).
However, the relationships between PLD1 and PLD2 activation and cellular responses
have not been well studied.

In contrast to LPARs, the protein growth factor receptors are receptor tyrosine kinases
(RTKs) and their activation stimulates signaling pathways, involving ERK, PI-3-K, PKC
and phospholipases (1, 22, 23). Receptors for LPA/S1P and growth factors (EGF and
PDGF) are co-expressed in many cell types including endothelial, smooth muscle and
epithelial cells (24-26). It is now becoming clearer that cross-talk between lipid
mediators, cytokines and growth factors regulate the amplitude and specificity of cellular
responses such as secretion, proliferation and differentiation. For instance several
agonists of GPCR, such as thrombin, angiotensin II, bradykinin, S1P and LPA not only
induce tyrosine phosphorylation of intracellular signaling proteins, but also transactivate
the growth factor receptors, EGF and PDGF (27-33). This transactivation amplifies
mitogenic signals from a variety of stimuli. However, relatively little is known regarding
the role and regulation of LPA-mediated transactivation of PDGF-Rs in mammalian cells.
In the present study, we investigated potential signaling pathways activated by LPA that regulate the transactivation of PDGF-Rβ in HBEpCs. LPA-induced phosphorylation of PDGF-Rβ in HBEpCs was sensitive to pertussis toxin. LPA also stimulated both PLD1 and PLD2 in HBEpCs; however, PLD2 but not PLD1 activation regulated LPA-mediated transactivation of PDGF-Rβ. Furthermore, over expression of wild type and catalytically inactive mutant of mPLD2 as compared to hPLD1, preferentially formed a complex with PDGF-Rβ in HBEpCs. Our results show for the first time that generation of PA by PLD2 activation is involved in LPA-induced PDGF-Rβ trans-activation and downstream signaling in human bronchial epithelial cells.
Materials and Methods

Materials

LPA was obtained from BIOMOL research Labs (Plymouth, PA). TPA, bovine serum albumin (fraction V), butan-1-ol, butan-3-ol and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant PDGF-BB, Tryphostins AG1296 and PTx were obtained from Calbiochem. Corp (La Jolla, CA). PBt and monoooleoyl LPA were purchased from Avanti Polar Lipid Corp (Alabaster, GA). \[^{32}\text{P}\]-Orthophosphate (carrier free) was obtained from New England Nuclear (Boston, MA). RedTaq DNA Polymerase was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). cDNA synthesis kit was purchased from Invitrogen (Carlsbad, CA) and total RNA isolation kit was obtained from QIAGEN (Valencia, CA). Polyclonal antibody against phospho-ERK was obtained from Cell Signaling (Beverly, MA). Antibodies for total PDGF-R\(\beta\) and phospho-PDGF-R\(\beta\) (Tyr.716) were purchased from Upstate (Lake Placid, NY) and antibodies against total-ERK were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phosphatidylcholine-specific PLD1, human N-terminal and internal as well as PLD2, mouse internal and N-terminal polyclonal antibodies were purchased from Biosource International (Camarillo, CA). BEBM Medium and supplement kit were purchased from BioWhittaker (Walkersville, Frederick MD). Primary human bronchial epithelial cells (HBEpCs), passage number two, were procured from Clonetics (San Diego, CA).
Cell Culture

Primary human bronchial epithelial cells (HBEpCs) were cultured in BEBM serum-free medium containing all the growth factors supplied by Clonetics (BEGM) at 37 °C in 5 % CO₂ / 95 % air. Cells were grown in to ~90% confluence in T-75 cm² vented flasks and subsequently propagated in 35 mm dishes. Cells were grown for 48 h without changing the medium before stimulation with LPA or PDGF-BB in conditioned BEBM medium (medium collected from cells cultured for 48 hours) containing 0.1% BSA. All experiments were carried out between passages 2 and 5.

Transfection and Viral Infection

For transient transfection, HBEpCs grown in 6-well plates (~60 confluence), were transfected with PDGF-Rβ or PLD2 plasmids (1 µg of cDNA was mixed with 6 µl of FuGene 6 / well in 1ml BMGM medium) according to the manufacturer’s recommendations. After 5 h, the transfection medium was aspirated and regular BEGM medium (2ml) was added and cells were incubated for 24-48 h. Infection of HBEpCs (~60% confluence) in 35 mm dishes was carried out with purified hPLD1 and mPLD2 adenoviral particles (50 p.f.u. / cell) in 1ml BEGM for 24h and the virus containing medium was replaced with complete BEGM for different time periods. For co-immunoprecipitation experiments, cells were infected with PLD1 or PLD2 adenoviral particles (50 p.f.u. / cell) for 3 h, and then transfected with PDGF-Rβ cDNA for 4 h. After transfection, the medium was removed, conditioned medium was added and cells were grown overnight before challenging with LPA or PDGF-BB.
Oligonucleotides for RT-PCR Analysis

**LPA-receptors:** Total RNA was isolated from HBEpCs using RNEasy kit (QIAGEN, Los Angeles, CA). cDNA was generated using cDNA synthesis kit according to manufacturer’s recommendation and subjected to PCR at 94 °C for 2 min following 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min, followed by 72°C for 10 min. The following oligonucleotides primers were used for RT-PCR analysis of LPA-Rs.

For LPA-1R, Forward: 5’-GTAATGGTGTTCTCTATGCTCAC-3’
   Reverse 5’-GGACAGCACACGTCTAGAAG-3’
For LPA-2R, Forward: 5’-GTCGAGCCTGCTTGTCTTC-3’
   Reverse: 5’-CCAGAGCAGTACCACCTG-3’
For LPA-3R, Forward: 5’-GGAATTGCCTCTGCAACATCT-3’
   Reverse: 5’-GAGTAGATGATGGGGTTCA-3’
For β-Actin: Forward: 5’-GACTACCTCATGAAGATC-3’
   Reverse: 5’-GATCCACATCTGCTGGAA-3’

The PCR products were separated by electrophoresis and primers used in these reactions yielded PCR products of 197 bp, 205 bp, 382 bp and 513 bp, respectively.

**PLD1 and PLD2:** PCR reactions were performed for 30 cycles at 94°C for 2 min followed by denaturation at 94°C for 1 min, annealing at 60°C for 2 min and elongation at 72°C for 3 min. The following oligonucleotides primers were used for RT-PCR.

PLD1 Forward: 5’-TGGGCTCACCATGAGAA-3’
PLD 1 Reverse: 5’-GTCATGCCAGGGCATCCGGGG-3’
PLD 2 Forward: 5’-TCCATCCAGGCCATTCTGCA-3’
PLD 2 Reverse: 5’-CGTTGCTCTCAGCCATGTCTTG-3’. PCR products were analyzed by agarose gel electrophoresis and the primers used in these reactions yielded products of 642 bp/ PLD1a; 528 bp/ PLD1b and 468 bp/ PLD2.

Preparation of Cell Lysates, Immunoprecipitation and Western Blotting

HBEpCs grown on 60 or 100 mm dishes (~ 90% confluence) were stimulated with LPA or PDGF-BB, rinsed three times with ice-cold PBS containing 1mM orthovanadate and lysed in RIPA buffer [20 mM Tris-HCl (pH 7.4), 1% Triton X-100 (v/v), 150 mM NaCl, 5 mM EDTA, 100 mM NaF and 1 mM Na3VO4]. Cell lysates were sonicated (3 x 15 sec) on ice, centrifuged at 5,000 g for 5 min at 4°C, and protein concentrations were determined with BCA protein assay kit (Pierce Chemical Co., Rockford IL) using BSA as standard. Equal amounts of protein (300-500 µg) were precleared with 20 µl Protein A / G agarose for 30 min at 4°C, centrifuged at 5,000 g for 10 min, supernatants were incubated with anti-PDGF-Rβ antibody (2 µg/mL) overnight at 4°C followed by addition of 40 µl protein A / G agarose and additional incubation for 2 h at 4°C. The cell lysates were centrifuged at 5,000 X g for 5 min, washed three times with ice-cold PBS containing 1 mM orthovanadate, protein-agarose complexes were dissociated by boiling for 5 min in 2x Lammeli buffer and samples were centrifuged in a microfuge at 5,000 g for 5 min. The supernatants were subjected to SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% (w/v) non-fat dry milk in TBST (25 mM Tris base, pH 7.4, 137 mM NaCl and 0.1% Tween 20) for 1 h and incubated with primary antibodies against PDGF-Rβ or phospho-PDGF-Rβ (1: 1000
dilution) in TBST overnight at 4 °C. The membranes were washed three times with TBST at 30 min intervals and then incubated with either mouse or rabbit horseradish peroxidase-conjugated secondary antibody (1: 3000 dilution) for 2-4 h at room temperature. The membranes were developed with enhanced chemiluminescence detection system according to manufacturer’s instruction and blots were quantified by densitometry and image analysis with Molecular Analyst software. Changes in PDGF-Rβ phosphorylation were expressed as fold change normalized to total cellular PDGF-Rβ protein.

**Measurement of Phospholipase D in Intact Cells**

HBEpCs in 35 mm dishes were labeled with $^{32}$P$_i$ orthophosphate (30 µCi / ml) BMGM medium for 18-24 h at 37 °C in 5% CO$_2$ and 95% air incubator. Cells were pre-treated with vehicle alone, or specific agents for specified times prior to challenge with LPA. Cells were then challenged with LPA in vehicle containing 0.1% BSA, or other agents and in the presence of 0.05% butan-1-ol. In some experiments, incubations were also carried out in the presence of 0.05% butan-3-ol that served as additional controls. Incubations were terminated by the addition of 1 ml of methanol: HCl (100:1 v/v), cells were scraped into glass tubes and lipids were extracted by the addition of 1 ml methanol: HCl (100:1 v/v), 2 ml of chloroform and 0.8 ml of 1M HCl (21). Formation of $[^{32}$P]PBt, as a result of PLD activation and transphosphatidylation of $[^{32}$P]PA, to butan-1-ol but not butan-3-ol, was separated from the total lipid extract by thin-layer chromatography on
1% potassium oxalate plates with the upper phase of ethyl acetate: 2, 2, 4-trimethyl pentane: glacial acetic acid: water (65:10:15:50 by volume) as the developing solvent system. Unlabeled PBt was added as carrier during separation of labeled lipids that were visualized by exposure to iodine vapor. Radioactivity associated with PBt was counted by liquid scintillation counting and all values were normalized to $10^6$ dpm in total lipid extract. $[^{32}\text{P}]$PBt formed in control and agonist challenged samples was expressed as dpm / dish or % control.

**Statistical Analysis**

Data are expressed as means of triplicates ± S.D. from at least three independent experiments. All results were subjected to statistical analysis using one-way ANOVA.
Results

Expression of LPA receptors in human bronchial epithelial cells: Recent cloning of GPCRs for LPA has identified three high affinity receptors, namely LPA-1, LPA-2 and LPA-3 (formerly known as Edg-2, Edg-4 and Edg-7) in mammalian cells and tissues. In the lung and human airway smooth muscle cells, mRNA for all three LPA receptors has been shown by RT-PCR (46). However, the relative expression and functional role of LPA-Rs in airway cells is unknown. Therefore, we determined the mRNA and protein expression of LPA receptor subtypes in primary cultures of human bronchial epithelial cells using RT-PCR, Western blotting and immunocytochemistry. As shown in Fig. 1A, mRNA for all the three LPA receptors was identified in HBEpCs. The presence of LPA-1 (50 KDa), LPA-2 (45 KDa) and LPA-3 (40 KDa) receptor proteins was also confirmed by Western blotting with specific antibodies to the three receptor proteins in HBEpCs (Fig. 1B) (provided by Glaxo Smith Kline, King of Prussia, PA). Furthermore, the Western blot results were complemented by immunocytochemical localization using LPA receptor antibodies. All the three LPA receptors were also detected in HBEpCs by immunocytochemistry (Fig. 1C). LPA-3 was barely detectable compared to LPA-1 and LPA-2 by immunofluorescent microscopy. Both LPA-1 and LPA-2 were not only localized at the plasma membrane, but were also distributed in the cytoplasm of the cell. The distribution of LPA-1 appeared punctated and located in the perinuclear membrane. The staining of LPA-3 was more pronounced at the perinuclear membrane as compared to the plasma membrane or cytoplasm distribution. These results indicate that LPA
receptors are not only present at the plasma membrane, but also distributed in the cytoplasm and perinuclear membranes.

**LPA-induced ERK1/2 Activation is sensitive to AG1296 and Pertussis Toxin in HBEpCs:** Previous studies have demonstrated stimulation of mitogenic signaling pathways for S1P, LPA and growth factors in human airway smooth muscle and epithelial cells (1, 21, 34 and 35). To further examine ERK activation, HBEpCs were challenged with LPA (1μM) or PDGF-BB (20 ng/ml) and phosphorylation of ERK was analyzed by Western blotting with phospho-specific antibodies against threonine/tyrosine residues. As shown in Figure 2, LPA and PDGF stimulated ERK1/2 phosphorylation by 2.2- and 1.8-fold, respectively. Enhanced phosphorylation of ERK, normalized to total ERK, was detected as early as 2 min of exposure to either LPA or PDGF-BB, while maximum phosphorylation (~ 3- to 4-fold) occurred at 15 min and declined thereafter (results not shown). As LPA and PDGF stimulated ERK, we next investigated the mechanisms of ERK activation. Treatment of cells with PD98059, a known inhibitor of MAPK/ERK kinase (MEK) blocked (> 95%) both LPA- and PDGF-BB mediated phosphorylation (results not shown). These results suggest that LPA- and PDGF-BB-induced activation of ERK is coupled to MEK signaling in HBEpCs. Pretreatment of cells with the PDGF-R selective tyrosine kinase inhibitor, tyrphostin AG1296, completely blocked PDGF-BB stimulated ERK phosphorylation whereas LPA activation of ERK was only partially blocked. This suggests that PDGF-R kinase activity is required for part of the LPA activation of the ERK cascade (Fig. 2). Previous studies showed that in Beas-2B cells, S1P-induced activation of ERK was sensitive to PTx (21) indicating the
involvement of G\textsubscript{i}-coupled S1P receptors. In HBEpCs pretreated with PTx (100ng/ml, 3 h), the LPA- stimulated ERK1/2 phosphorylation was attenuated by about 95% (Fig.3). These results demonstrate that LPA receptors are coupled to PTx sensitive G\textsubscript{i} in the ERK signaling cascade.

**LPA Induction of PDGF-R\textbeta{} Phosphorylation is coupled through G\textsubscript{ai}:** The inhibition of LPA-induced ERK activation by AG1296 suggests that LPA-R stimulation results in PDGF-R transactivation. Therefore, we assessed PDGF-R\textbeta{} phosphorylation in response to LPA. HBEpCs were challenged with LPA and PDGF-R\textbeta{} phosphorylation was studied by Western blotting with phospho-specific PDGF-R\textbeta{} antibody (specific for tyrosine 716). As shown in Fig. 4, stimulation of HBEpCs with varying concentrations of LPA (0.1-5 \textmu{}M) increased PDGF-R\textbeta{} tyrosine phosphorylation. Enhanced phosphorylation of PDGF-R\textbeta{} after 15 min of exposure to 0.1 \textmu{}M LPA (~4 fold increase over control) increased to ~7 fold with 0.5 \textmu{}M LPA (Fig. 4). Furthermore, LPA stimulated PDGF-R\textbeta{} phosphorylation in a time-dependent manner (Fig. 5). Enhanced tyrosine phosphorylation of PDGF-R\textbeta{} (~2 fold) was detected within 2 min of challenge to LPA while maximum phosphorylation (~10 fold) was observed at 5 min and declined thereafter up to 30 min (Fig. 5). In addition to tyrosine 716, PDGF-R\textbeta{} is also tyrosine phosphorylated at other sites by PDGF. We therefore compared the Western blots of phospho-specific tyrosine 716 antibodies with immunoprecipitation of PDGF-R\textbeta{} and Western blotting with anti-phosphotyrosine antibody. HBEpCs were transfected with PDGF-R\textbeta{} wild type plasmid for 18 h and cells were stimulated with LPA (1 \textmu{}M) for different times. Cells lysates (1.0 mg protein) were immunoprecipitated with PDGF-R\textbeta{} antibody and precipitates were
analyzed by Western blotting with anti-phospho-tyrosine antibody. As shown in Fig. 5, over expression of cells with wild type PDGF-Rβ plasmid and stimulation with LPA provided a similar time-dependent tyrosine phosphorylation of PDGF-Rβ. Phosphorylation of PDGF-Rβ was detected after 5 min of exposure to 100 nM LPA (≈ 3 fold increase over control), which increased to ≈ 7 fold with 500 nM LPA and plateaued at higher concentrations of LPA (1 and 5 μM). These results clearly demonstrate phosphorylation of PDGF-Rβ by LPA in HBEpCs.

To further ascertain the role of LPA-R stimulation by LPA in trans-activation of PDGF-Rβ, we investigated the effect of PDGF-R kinase inhibitor, tyrphostin AG1296 on tyrosine phosphorylation of PDGF-Rβ. The LPA-induced tyrosine phosphorylation of PDGF-Rβ (≈ 6 fold increase over control in 10 min) was attenuated by tyrphostin AG1296 in a dose-dependent manner with a ≈ 50% inhibition at 1 μM AG1296 (Fig. 6 A). However, the basal phosphorylation of PDGF-Rβ, in the absence of LPA, was not drastically altered by AG1296 (Fig. 6A). As expected, pretreatment of cells with AG1296 (1 μM) for 1 h also abolished the PDGF-BB (20 ng/ml) mediated PDGF-Rβ phosphorylation (Fig. 6B). These results provide further evidence that LPA causes activation of the PDGF-Rβ in HBEpCs.

LPA receptors are coupled to a variety of heterotrimeric G proteins (7). Previously, we and others showed that Gαi coupling played an important role in S1P- and LPA-mediated signal transduction in Beas-2B cells (21) and fibroblasts (36). To determine the contribution of Gαi in LPA-induced PDGF-Rβ trans-activation, HBEpCs were pretreated
with PTx (100 ng / ml, 12 h) prior to stimulation with LPA (1 µM) for 15 min. This treatment dramatically inhibited tyrosine phosphorylation of PDGF-Rβ (~70% inhibition compared to stimulation by LPA alone), whereas, PTx had no effect on basal phosphorylation of PDGF-R (Fig.7). As a control, we established that PTx had no significant effect on PDGF-BB-mediated tyrosine phosphorylation of PDGF-Rβ (results not shown). These results suggest that LPA-induced transactivation of PDGF-Rβ is partly regulated by coupling of an LPA-R to G_i.

**Role of PLD in LPA-Induced PDGF-Rβ Transactivation:** The internalization of growth factor receptors in response to EGF or PDGF involves endocytosis (37, 38). Since PLD has been implicated in protein trafficking and membrane fusion, we hypothesized that LPA-mediated PLD stimulation and PA generation may be involved in PDGF-Rβ transactivation in HBEpCs. RT-PCR revealed presence of mRNA for PLD1 and PLD2 while Western blotting with specific antibodies confirmed expression of PLD1 (116 KDa) and PLD2 (100 KDa) proteins in primary cultures of HBEpCs (Fig. 8). As shown in Table-1, exposure of [32P]-labeled HBEpCs to LPA (1 µM) for 10 min, in the presence of 0.05% butan-1-ol, significantly enhanced [32P]PBt accumulation, an index of PLD activation (39). However, under similar incubation condition, PDGF-BB (50 ng/ml) did not enhance [32P]PBt formation (Table-1). Furthermore, the PDGF-R kinase inhibitor, AG1296, had no significant effect on LPA- or TPA-induced [32P] PBt formation (Table-1). These results demonstrate that activation of PLD by LPA is not mediated through PDGF-R in HBEpCs.
The role of LPA-induced PLD activation in transactivation of the PDGF-Rβ was investigated by using the ability of primary, but not secondary or tertiary alcohols to divert PA formation to accumulation of phosphatidylylalcohol. HBEpCs were stimulated with LPA (1 μM) for 15 min in the absence or presence of 0.1% butan-1-ol, or butan-3-ol. As shown in Fig. 9, butan-1-ol, but not butan-3-ol, blocked LPA-induced PDGF-Rβ phosphorylation (LPA, ~ 5 fold increase compared to vehicle; butan-1-ol plus LPA, ~ 3 fold change) without affecting the basal phospho-PDGF-R levels. These results demonstrate the involvement of PLD in LPA-induced transactivation of PDGF-Rβ in HBEpCs.

**Role of PLD2 rather than PLD1 in LPA-induced PDGF-Rβ phosphorylation:** To further investigate the involvement of PLD isoenzymes in LPA-induced PDGF-Rβ transactivation, we over expressed wild type and catalytically inactive mutants of hPLD1 and mPLD2. HBEpCs (~50% confluence) were infected with the adenoviral constructs (50 p.f.u. / cell) for 12, 24 and 48 h and cell lysates were analyzed for enhanced expression of PLD1 and PLD2 proteins by Western Blot analysis using N-terminal plus internal anti PLD1, or PLD2 antibodies. As shown in Fig. 10A, infection of HBEpCs with adenoviral constructs increased the expression of PLD1 and PLD2 proteins in a time-dependent fashion with expression of the proteins seen as early as 12 h and maximum expression observed at 48 h after infection. The functional roles of hPLD1 and mPLD2 over expression were tested by determining which of the isoenzymes of PLD are activated by LPA in HBEpCs. In both control and vector-infected cells, LPA (1 μM) stimulated \[^{32}P\]PBt formation by 4- to 5-fold compared to an increase of 7.5-fold in cells that over expressed hPLD1. Over expression of mPLD2 increased basal PLD activity by
about 3.6-fold and this activity was increased further by about 5-fold by stimulation of cells with LPA (Fig. 10B). By contrast, over expression of catalytically inactive mutant of mPLD2 (K758R), but not hPLD1 (K898R), partially blocked LPA-mediated $[^{32}P]$PBt accumulation (Fig. 10B). These results show that LPA stimulates PLD2 $>$ PLD1 and the catalytically inactive mutant of mPLD2 attenuated LPA-mediated $[^{32}P]$-PBt formation in HBEpCs.

We then used these cells that expressed wild type and catalytically inactive mutants of hPLD1 and mPLD2, to study their respective roles in activation of the PDGF-R$\beta$. As shown in Fig. 11A, over expression of either wild type hPLD1 or its mutant K898R had no effect on LPA-mediated PDGF-R phosphorylation. However, in cells infected with cDNA for wild type mPLD2, the basal ($\sim$1.5 fold change) as well as LPA-mediated ($\sim$3.0 fold change) phosphorylation of the PDGF-R$\beta$ were significantly higher compared to the cells infected with empty vector (Fig. 11B). Over-expression of the catalytically inactive mutant of mPLD2 attenuated both the basal and LPA-induced phosphorylation of PDGF-R$\beta$ (Fig. 11B). These results establish the involvement of PLD2, rather than PLD1 in LPA-mediated PDGF-R trans-activation in HBEpCs.

To study this action further, we investigated possible interaction between PLD1, PLD2 and PDGF-R$\beta$ in the absence, or presence of LPA. HBEpCs were infected with adenoviral constructs of wild type hPLD1, mPLD2 and catalytically inactive mutants hPLD1 (K898R) and mPLD2 (K758R) for 12 h followed by a second transfection with wild type PDGF-R$\beta$ for another 24 h. Immunoprecipitation of the PDGF-R$\beta$ from vehicle
and LPA challenged cells resulted in co-immunoprecipitation of wild type mPLD2 and catalytically inactive mutant mPLD2-K758R expressed proteins compared to vector control (Fig. 12A). Relative to mPLD2, the amount of hPLD1 that co-immunoprecipitated with PDGF-Rβ was almost negligible in vehicle and LPA stimulated HBEpCs (Fig. 12A). The Western blots for PLD1 and PLD2 in PDGF-Rβ immunoprecipitates were carried out simultaneously and for the same exposure times to ensure identical development of the samples. In contrast to co-immunoprecipitation with PDGF-Rβ, a comparable expression of PLD1 and PLD2 proteins was observed in total cell lysates from control and LPA treated cells (Fig. 12B). Although stimulation of cells with PDGF-BB increased tyrosine phosphorylation of PDGF-Rβ, over-expression of either the wild type mPLD2 or catalytically inactive PLD2 mutant had no effect on PDGF-Rβ phosphorylation (data not shown). Presumably, PDGF did not increase association of PLD2 with PDGF-Rβ. These results show that in HBEpCs, PDGF-BB mediated phosphorylation of PDGF-Rβ is independent of PLD stimulation in HBEpCs and PLD2, but not PLD1 forms a physical complex with PDGF-Rβ which is independent of the catalytic activity of PLD2.
LPA mediates cellular responses through its GPCRs. Many responses to LPA such as proliferation, anti-apoptotic and tumor invasiveness are mediated by signaling pathways of protein kinase C, Src kinase, ERK, phospholipases and Ca\(^{2+}\) (40). Many GPCRs, including those for platelet-activating factor, S1P, LPA, thrombin, insulin growth factor-1, chemokine CXCR-1/2, \(\beta_2\) –adrenergic agonists and angiotensin-II (28,30,31,41-45) activate ERK1/2 involving transactivation of receptor tyrosine kinases (RTKs). Earlier studies showed that LPA induces protein tyrosine phosphorylation in fibroblasts and exhibits growth factor like properties via ERK activation (36). The role of LPA and its receptors in bronchial epithelial cell signal transduction and cellular responses has not been well characterized. Here we report for the first time that LPA transactivates PDGF-R in human bronchial epithelial cells resulting in stimulation of ERK1/2. Furthermore, we demonstrated a novel mechanism that this trans-regulation of the PDGF-R by LPA is mediated by coupling to G\(_i\) and PA generated by PLD2, but not PLD1.

LPA-1, LPA-2 and LPA-3 receptors are present in the lung (46). RT-PCR studies have confirmed mRNA for all the three receptors in isolated human aortic smooth muscle cells (47). In human bronchial epithelial cells, we detected the mRNA for all the three receptors by RT-PCR, protein expression by Western blotting with LPA-R specific antibodies and immunocytochemistry (Fig.1). Interestingly, the immunocytochemical analysis revealed that the LPA receptors apparently have differential sub-cellular localization. LPA receptors were detected not only on the plasma membrane, but also, in the cytoplasm, endoplasmic reticulum and perinuclear location. Western blot analysis
using antibodies directed against internal domains of LPA1-3 of the LPA1-3 R revealed considerable specificity with LPA-2 antibody exhibiting a single band corresponding to the expected weight of each receptor (data not shown). The intracellular distribution of LPA-Rs indicates that they may be targets of the LPA generated following agonist stimulation (48). More than one LPA receptor could be involved in LPA-induced ERK1/2 activation. Further studies on the types of LPA-Rs that are coupled to different GPCRs and native LPA receptor localization will help in understanding intracellular targets of LPA and its action.

LPA receptors are coupled to heterotrimeric G proteins: G\textsubscript{i}, G\textsubscript{q/G11} and G\textsubscript{12/13}, increasing the complexity in signaling pathways inducing multiple cellular responses. In HASMs, ERK activation by LPA was blocked by PTx treatment (1, 49). In fibroblasts, LPA stimulated cell growth and decreased in cAMP was sensitive to PTx suggesting coupling of LPA receptors to G\textsubscript{i} (8). Similarly, LPA-mediated activation of Ras/MAPK cascade and cell migration occurred in a PTx-sensitive manner with the possible involvement of G\textsubscript{b\gamma} subunits (10). However, in several mammalian cells, LPA-induced stimulation of phospholipase C was PTx-insensitive suggesting LPA-R coupling to G\textsubscript{q} (50). A role for the G\textsubscript{12/13} family of heterotrimeric G proteins in LPA-induced actin stress fiber and focal adhesions has been described in quiescent Swiss 3T3 fibroblasts (13). Our present results demonstrate that LPA-induced transactivation of PDGF-R\textbeta and ERK1/2 stimulation is PTx-sensitive suggesting involvement of G\textsubscript{i} protein dependent signaling responses in HBEpCs (Figs. 3 and 7). The LPA-R mediated ERK1/2 activation appears to be secondary to transactivation and phosphorylation of PDGF-R\textbeta since the PDGF-R
tyrosine kinase inhibitor, AG 1296 (51,52), partially blocked LPA-induced ERK stimulation (Fig. 2). There are several reports demonstrating transactivation of EGF-R by LPA (31, 50, 53-57). Tyrphostin AG 1478, an inhibitor of EGF-R kinase, blocked LPA-induced EGF-R phosphorylation suggesting transactivation of EGF-R by LPA in HBEpCs (results not shown). Furthermore, pretreatment of HBEpCs with AG 1296 plus AG 1478 almost completely attenuated LPA-mediated phosphorylation of ERK1/2 (results not shown) compared to the partial block with AG1296 alone (Fig. 2). These results suggest that in HBEpCs, transactivation of both EGF-R and PDGF-R by LPA plays a major role in the activation of ERK1/2. Further studies are required to evaluate the mechanisms for transactivation of the EGF-receptor with respect to ERK signaling.

LPA-R induced EGF-R transactivation involves the matrix metalloproteinase dependent proteolytic processing of membrane-anchored pro-ligands of EGF receptor (31). However, in our experiments, LPA-induced trans-activation of PDGF-R was not attenuated by the non-specific matrix metalloproteinase inhibitor, GM 6001, in HBEpCs (results not shown). Furthermore, inhibitors of PKC such as bisindolylmaleimide and Gö 6738, failed to block LPA- induced PDGF-R phosphorylation suggesting that PKC is not involved. LPA-induced transactivation of PDGF-Rβ was attenuated by butan-1-ol, but not butan-3-ol in HBEpCs (Fig. 9) demonstrating a role for PLD in transactivation of PDGF-R. Using formation of $[^{32}P]$PBt in the presence of butan-1-ol, as an index of PLD activation, we demonstrated that LPA but not PDGF-BB, activated PLD in HBEpCs (Table 1). Additionally, infecting cells with adenoviral constructs of wild-type hPLD1
and mPLD2 demonstrated that LPA rapidly and transiently activated both PLD1 and PLD2 in HBEpCs.

Earlier, we showed that S1P activated both PLD1 and PLD2 in Beas-2B bronchial epithelial cells (21). In the present work, the participation PLD2, but not PLD1, in LPA mediated transactivation of PDGF-R was established by the demonstration that over-expression of mPLD2-K758R, but not the hPLD1-K898R mutant, blocked LPA-induced phosphorylation of PDGF-Rβ. The two PLD mutants had no effect on PDGF-BB induced phosphorylation of PDGF-R confirming that PLD was not activated downstream of PDGF binding to PDGF-R in HBEpCs. These results provide the first direct evidence for activation of both PLD1 and PLD2 in HBEpCs and for the participation of PLD2 but not PLD1, in LPA-induced transactivation of PDGF-R. Our results (Fig. 12A) show that wild-type and catalytically inactive mutant of mPLD2 co-immunoprecipitated with PDGF-Rβ to a greater extent compared to PLD1 before and after stimulation of HBEpCs with LPA. Also, mutation of mPLD2 at a single amino acid residue at 758 from K to R had no appreciable effect on its interaction with the PDGF-Rβ (Fig. 12A). However, this mutation at the catalytic site of mPLD2 almost completely reversed LPA-induced transactivation of PDGF-Rβ. Earlier studies using human embryonic kidney fibroblasts (HEK293) transfected with wild type PLD1 or PLD2 demonstrated that EGF stimulated PLD1 activity to a greater extent than PLD2, but only PLD2 was associated with EGF-R (58). Furthermore, PLD2 was tyrosine phosphorylated upon EGF-R activation by EGF (59). However, in HBEpCs, PLD was not activated by PDGF (Table 1), or EGF (results not shown) and it is unclear if LPA enhanced tyrosine phosphorylation of PLD2. Studies
with rat 3Y1 fibroblasts, over-expressing the EGF-R established a role for both PLD1 and PLD2 in EGF-induced receptor degradation suggesting that PLD signaling is involved in receptor endocytosis (60).

Our work, therefore, established a novel mechanism by which PLD2 participates in the transactivation of PDGF-Rβ. We have not yet elucidated the mechanisms for activation other than to establish that PLD2 interacts physically with PDGF-Rβ and that the formation of PA is required. We have also not investigated the details of how PLD2 is activated by exogenous LPA, but this involves a receptor, probably LPA-1 that is coupled to Gαi. Src kinase plays a central role in GPCR signaling by transactivating the EGF-R or VEGF-R (Flk-1/KDR) (61). For example, adrenergic receptor mediated EGF-R phosphorylation and S1P-induced Flk-1/KDR transactivation (32, 62) was sensitive to Src kinase inhibitors, PP1/PP2 (63). Our work with endothelial cells established that Src can be upstream of activation of PLD1 and PLD2 by diperoxovanadate (64). Therefore, such an LPA-induced activation of Src in HBEpCs could mediate the PLD2-induced activation of PDGF-Rβ. It is also significant that the PDGF-Rβ can be tethered to GPCRs, including S1P-1, thus providing an integrative signaling pathway (33). Our present results suggest that PLD2 could be a component of such a complex.

In summary, we demonstrate for the first time that LPA mediates trans-activation of PDGF-R in HBEpCs through PLD and PA production. Although LPA activates both PLD1 and PLD2, PLD2, but not PLD1, regulates PDGF-R phosphorylation and activation. Also, LPA increases PLD2 association with PDGF-Rβ. Our results, therefore,
demonstrate that PLD2 is activated upstream of PDGF-Rβ activation. PDGF signaling is involved in cell proliferation and tumorigenesis, as well as lung development. Our work establishes that LPA through stimulating PLD2 activity plays an important role in activating the PDGF-Rβ and therefore ERK. LPA-induced transactivation of the PDGF-R could, therefore, be involved in modulating airway remodeling and lung function.
Acknowledgment

We thank Drs. Henry Sarau and Kristen Belmonte from Glaxo Smith Kline for providing the LPA-1, LPA-2 and LPA-3 receptor antibodies. The technical assistance of Tonya Watkins and Dong Hong He is appreciated. We also thank Dr. Evegnia Berdyshev for critical reading of the manuscript. This work was supported by the National Institutes of Health grant HL 71121 to V.N. and by a CIHR grant (MOP 10504) to DNB.
FIGURE LEGENDS

Figure 1: Detection of LPA receptors by RT-PCR, Western blotting and Immunocytochemistry. A, Total RNA was extracted from primary HBEpCs and transcription of the genes encoding LPA receptors (1, 2 and 3) was assessed by RT-PCR (- indicates in the absence of RNA and + indicates in the presence of 2 µg RNA) with primers to the indicated receptors and β-actin. RT-PCR products were visualized by ethidium bromide staining after separation on agarose gels. B, Cell lysates (30 µg proteins) were subjected to SDS-PAGE and analyzed by Western blotting with LPA-1 or LPA-2 or LPA-3 receptors. Each Western blot is representative of three independent experiments. C, HBEpCs grown on cover slips to ~90% confluence were subjected to immunostaining with LPA-receptor antibodies and examined by fluorescent microscopy. Each immunofluorescence image is representative of three separate experiments.

Figure 2: Effect of tyrphostin AG1296 on LPA- and PDGF-BB-mediated ERK phosphorylation. HBEpCs (passage 2, ~ 90% confluence in 35 mm dishes) were pretreated with BEBM or BEBM plus AG1296 (5 µM) for 1 h and then stimulated with A, LPA (1 µM) or B, PDGF-BB (20 ng/ml) for 15 min. Cell lysates (30 µg protein) from A and B were subjected to SDS-PAGE and Western blotted with phospho-specific and pan-ERK antibodies. Values are means ± S.D from three independent experiments in triplicate and fold changes in ERK phosphorylation were normalized to total ERK. The histograms are shrunk horizontally to fit under the blots.
Figure 3: Pertussis Toxin blocks LPA-induced ERK phosphorylation. HBEpCs (Passage 2, ~90% confluence in 35 mm dishes) were pretreated with pertussis toxin (100 ng/ml) for 3 h. Cells were challenged with LPA (1 µM) for 15 min, then cell lysates were subjected to SDS-PAGE and Western blotted with phospho-specific and pan-ERK antibodies. Values are means ± S.D. of three independent experiments in triplicate. Fold changes in ERK phosphorylation were normalized to total ERK.

Figure 4: Dose-dependent phosphorylation of PDGF-Rβ by LPA. HBEpCs (Passage 3, ~90% confluence) in 35 mm dishes were challenged with increasing concentrations of LPA as indicated for 5 min, and cell lysates were analyzed by Western blotting with phosho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies as described in “Experimental Procedures” section. Values are means ± S.D. from three independent experiments and fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ. The histogram is expanded vertically.

Figure 5: LPA stimulates phosphorylation of PDGF-Rβ. A, HBEpCs (Passage 3, ~80% confluence in 35 mm dishes) were transfected with PDGF-Rβ plasmid DNA for 4 h, then medium containing DNA-transfection reagent complex was replaced with conditioned BEBM medium, cells were cultured for another 24 h and challenged with 1 µM LPA for various times as indicated. To cell lysates (1 mg protein), 2 µg/ml anti PDGF-Rβ antibody was added, incubated for 18 h at 4 °C followed by addition of sepharose A/G (20 µl) and additional incubation for 2 h at 4°C, the immunocomplex was
centrifuged at 5000 x g for 10 min and pellet was washed three times with ice-cold RIPA buffer containing 1 mM orthovanadate and protease inhibitors. A, the immunoprecipitates were subjected to SDS-PAGE, transferred onto PVDF membrane and probed with anti-tyrosine antibody. Membranes were stripped and re-probed with anti-PDGF-Rβ antibody for total PDGF-Rβ. B, cell lysates were also analyzed by Western Blotting with phospho-specific anti-PDGF-Rβ antibody (against tyrosine residue 716). Values are means ± S.D. from three independent experiments and fold changes in PDGF-R phosphorylation were normalized to total PDGF-Rβ. The histograms are shrunk horizontally to fit under the blots.

Figure 6: AG 1296 attenuates LAP- and PDGF-BB mediated PDGF-Rβ phosphorylation. HBEpCs (Passage 2, ~80% confluence) in 35 mm dishes were treated with varying concentrations of tyrphostin AG 1296 (A) or 1 µM AG 1296 (B) for 1 h. Subsequently the cells challenged with LPA (1 µM) as in (A) or PDGF-BB (20 ng/ml) as in (B) for 5 min in BEBM with 0.1% BSA. Cell lysates were subjected to SDS-PAGE and Western blotting with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are means ± S.D. of triplicate determinations and fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ.

Figure 7: PTx blocks LPA-induced PDGF-Rβ transactivation. HBEpCs (Passage 3, ~90% confluence) in 35 mm dishes were treated with PTx (100 ng/ml for 12 h) prior to challenge with LPA (1 µM) for 15 min. Cell lysates were prepared and analyzed for PDGF-Rβ phosphorylation with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-
Rβ antibodies. Values are means ± S.D. of three independent experiments and fold changes in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ.

**Figure 8: Detection of PLD1 and PLD2 in HBEpCs by RT-PCR and Western blotting.** A, Total RNA was extracted from primary HBEpCs and transcription of the genes encoding PLD1 and PLD2 was assessed by RT-PCR (- indicates in the absence of RNA and + indicates in the presence of 2 µg RNA) with primers as indicated under Materials and Methods. RT-PCR products were visualized by ethidium bromide staining after separation on agarose gels. B, Cell lysates (40 µg protein) were subjected to SDS-PAGE on 6% gels and analyzed by Western blotting with PLD1 (Internal + N-terminal antibodies, 1: 500 dilution) and PLD2 (Internal + N-terminal antibodies, 1:1000 dilution). The Western blot is representative of three independent experiments.

**Figure 9: Butan-1-ol, but not butan-3-ol, attenuates LPA-induced PDGF-Rβ phosphorylation.** HBEpCs (Passage 2, ~80 % confluence) in 35 mm dishes were pretreated with BEBM or BEBM plus butan-1-ol (0.1%) or butan-3-ol (0.1%) for 15 min and then challenged with LPA (1 µM) in 0.1% BSA for an additional 15 min. Cell lysates (20-30 µg protein) were subjected to SDS-PAGE and Western Blotting with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are means ± S.D. from three independent experiments and fold changes PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ in total cell lysates. * P < 0.05 compared with vehicle control; * P < 0.05 compared with LPA treatment.
Figure 10: Effects of overexpression of wild type and catalytically inactive mutants of hPLD1 and mPLD2 on LPA-induced $[^{32}P]P_{Bt}$ formation. A, HBEpCs (~ 50% confluence in 35 mm dishes) were infected with empty vector or adenoviral vectors containing cDNA for wild type hPLD1 or mPLD2 or mutant forms of hPLD1 or mPLD2 (50 p.f.u. / cell; 1.5 x $10^5$ cells per dish) in complete BEBM medium for 12, 24 and 48 h. Cell lysates were prepared as described in “Experimental Procedures” and subjected to SDS-PAGE and Western blotting with internal plus N-terminal PLD1 and PLD2 antibodies. B, HBEpCs (~ 50% confluence in 35 mm dishes) were infected as described above for 24 h and subsequently labeled with $^{32}P_i$ for 18 h. Cells were challenged with BEBM or BEBM plus LPA (1 $\mu$M) containing 0.1 % BSA and 0.05% butan-1-ol for 15 min. Lipids were extracted under acidic conditions and $[^{32}P]P_{Bt}$ formed was quantified after separation of the total lipid extracts by TLC. Values are means ± S.D. of triplicate determinations from three independent experiments and normalized to $10^6$ d.p.m. in total lipid extracts. * $P < 0.05$ compared with vehicle control/vector; ** $P < 0.05$ compared with LPA treatment in vector infected cells; *** $P > 0.05$ compared with LPA treatment in vector infected cells; **** $P < 0.05$ compared with vector infected cells.

Figure 11: Effects of over expression of wild type and mutants of hPLD1 and mPLD2 on LPA-induced PDGF-Rβ phosphorylation. A, HBEpCs (~ 50% confluence in 35 mm dishes) were infected with adenoviral vectors containing cDNA for wild type hPLD1 or mutant hPLD1 (50 p.f.u. / cell, 1.5 x $10^5$ cells) for 48 h. Cells were challenged with BEBM or BEBM plus LPA (1 $\mu$M) containing 0.1% BSA for 5 min. B, HBEpCs were infected with vector or wild type mPLD2 or mutant mPLD2 for 48 h before
challenging with LPA (1 µM) for 5 min as described above. Cell lysates were subjected to SDS-PAGE and Western blotting with phosho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are means ± S.D. of three independent experiments in triplicate. Fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ in the cell lysates.

**Figure 12:** Effects of over expression of wild type and mutant mPLD2 on co-immunoprecipitation of PDGF-Rβ with PLD2 after LPA treatment: A, HBEpCs (Passage 3, ~ 50% confluence in 35 mm dishes) were infected with adenoviral vectors containing cDNA for wild-type or mutant mPLD2 (50 p.f.u. / cell, 1.5 x 10⁵ cells) for 24 h. Cells were then transfected with wild-type PDGF-Rβ plasmid with FuGene 6 for 5 h. Then the medium was replaced with fresh BEBM and cells cultured for an additional 24 h, cells were challenged with BEBM or BEBM plus LPA (1 µM) in 0.1 % BSA for 5 min. Cell lysates (1 mg protein / ml) were subjected to immunoprecipitation under non-denaturing conditions with PDGF-Rβ antibody for 18 h in the presence of Sepharose A/G (20 µl/mg protein). The PDGF-Rβ immunoprecipitates were subjected to SDS-PAGE and Western blotting with PDGF-Rβ and internal plus N-terminal PLD2 antibodies. B, Cell lysates (20 µg protein) were subjected to SDS-PAGE and Western blotting with internal plus N-terminal PLD2 antibody.


Table – 1
Effect of AG 1296 on PLD Activation by LPA, PDGF-BB and TPA in HBEpCs.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Treatment</th>
<th>$[^{32}P] \text{PBt} – \text{formed (dpm)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>550 ± 71</td>
</tr>
<tr>
<td>Vehicle</td>
<td>LPA (1µM)</td>
<td>3081 ± 382</td>
</tr>
<tr>
<td>Vehicle</td>
<td>PDGF-BB (50 ng/ml)</td>
<td>682 ± 21</td>
</tr>
<tr>
<td>Vehicle</td>
<td>TPA (25 ng/ml)</td>
<td>3708 ± 234</td>
</tr>
<tr>
<td>AG 1296 (10 µM)</td>
<td>Vehicle</td>
<td>422 ± 33</td>
</tr>
<tr>
<td>AG 1296 (10 µM)</td>
<td>LPA (1 µM)</td>
<td>2796 ± 208</td>
</tr>
<tr>
<td>AG 1296 (10 µM)</td>
<td>PDGF-BB (50 ng/ml)</td>
<td>538 ± 46</td>
</tr>
<tr>
<td>AG 1296 (10 µM)</td>
<td>TPA (25 ng/ml)</td>
<td>3432 ± 136</td>
</tr>
</tbody>
</table>

HBEpCs were labeled with $^{32}$Pi (20 µCi / ml) in BEBM serum free medium for 18 h. The radioactive medium was aspirated and cells were pretreated with AG 1296 (10µM) for 1h prior to addition of either LPA (1µM) or PDGF-BB (50 ng / ml) or TPA (25 nM) for 15 min in the presence 0.1% BSA and 0.05% butan-1-ol. Lipids were scraped under acidic conditions with methanol: HCl and extracted with chloroform as described in Materials and Methods. The formation of $[^{32}P]\text{PBt}$, an index of PLD activation, was quantified by scintillation counting after separation of the labeled lipids by thin-layer chromatography using 1% potassium oxalate plates developed with the upper phase of ethyl acetate: iso-octane: glacial acetic acid: water (65:10:15:50, by vol.). Values are means ± SD of three independent experiments in triplicate. Counts (dpm) in $[^{32}P]\text{PBt}$ were normalized to $1 \times 10^6$ dpm in total lipid extracts.
Figure 1

A. RT-PCR

B. Western Blotting

C. Immuno-cytochemistry
Figure 2

A

Phospho-ERK

Total-ERK

LPA (1µM)  -  -  +  +
AG1296 (20 µM)  -  +  -  +
PDGF-BB (20 ng/ml)  -  -  -  -

B

44 KDa

42 KDa

44 KDa

ERK phosphorylation (fold change)

vehicle AG1296 LPA LPA+ AG1296

ERK phosphorylation (fold change)

vehicle AG1296 PDGF-BB PDGF-BB+ AG1296
Figure 3

Phospho-ERK

Total ERK

LPA (1 µM) - + - +
PTx (100ng/ml) - - + +

ERK phosphorylation (fold change)

- 0 1 2 3

vehicle LPA PTx LPA+PTx

* ** ***
Figure 4

Phospho-PDGF-Rβ (fold changes) in response to varying concentrations of LPA (µM).

- **Phospho-PDGF-Rβ**
  - LPA (µM): 0, 0.1, 0.2, 0.5, 1, 5
  - Phospho-PDGF-Rβ levels increase with LPA concentration.

- **Total-PDGF-Rβ**
  - LPA (µM): 0, 0.1, 0.2, 0.5, 1, 5
  - Total-PDGF-Rβ levels remain relatively constant across different LPA concentrations.

180 KDa marker indicates protein bands.

Bar graph showing fold changes in Phospho-PDGF-Rβ expression with LPA concentrations.
Figure 5

A

IP: PDGF-Rβ
IB: Phospho-tyrosine
IB: Total-PDGF-Rβ

Time (min) 0 2 5 15 30

B

IB: phospho-PDGF-Rβ
IB: Total-PDGF-Rβ

180 KDa

Tyrosine Phosphorylation (fold change)

Phospho-PDGF-Rβ (fold change)

Time (min) 0 2 5 15 30

180 KDa
Figure 6

A

**Phospho-PDGF-Rβ**

**Total-PDGF-Rβ**

LPA (1 µM)

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG1296 (µM)</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

B

**Phospho-PDGF-Rβ**

**Total-PDGF-Rβ**

180 KDa

PDGF-BB (20 ng/ml)

AG1296 (1 µM)

**Phospho-PDGF-Rβ** (fold change)

vehicle

PDGF

AG1296

PDGF

AG1296

**Phospho-PDGF-Rβ** (fold change)

0 0.2 1.0

AG1296 (µM)

**Phospho-PDGF-Rβ** (fold change)

* *

**Phospho-PDGF-Rβ** (fold change)

vehicle

PDGF

AG1296

PDGF

AG1296
Figure 7

Phospho-PDGF-Rβ

Total PDGF-Rβ

Vehicle  LPA  PTx  LPA+PTx

180 KDa

180 KDa

Phospho-PDGF-Rβ (fold change)

vehicle  LPA  PTx  LPA+PTx

*  

**
Figure 8

A)

PLD1a  
PLD1b

RT  +  -  +  -
    PLD1  PLD2  M

B)

116 KDa  
100 KDa

PLD1  PLD2
Figure 9

Phospho-PDGF-Rβ

Total PDGF-Rβ

LPA (1 µM) - + - + - +
Butan-1-ol - - + + - -
Butan-3-ol - - - - + +

Phospho-PDGF-Rβ (fold Change)

LPA (1 µM) - + - + - +
Butan-1-ol - - + + - -
Butan-3-ol - - - - + +

180 KDa

* ** ***
Figure 11

A

IB: Phospho-PDGF-Rβ

IB. Total PDGF-Rβ

Vector | PLD1b | PLD1b-mu.

LPA (1 µM) - + - + - +

Phospho-PDGF-Rβ (fold change)

LPA (1 µM) - + - + - +

B

IB: Phospho-PDGF-Rβ

IB. Total PDGF-Rβ

Vector | mPLD2 | mPLD2-mu.

LPA (1 µM) - + - + - +

Phospho-PDGF-Rβ (fold change)

LPA (1 µM) - + - + - +
Figure 12

A

**IP: PDGF-Rβ**

- IB: PDGF-Rβ
  - 180 KDa
- IB: PLD2
  - 100 KDa
- IB: PLD1
  - 116 KDa

B

**Cell Lysates**

- IB: PLD2
  - 100 KDa
- IB: PLD1
  - 116 KDa

<table>
<thead>
<tr>
<th>LPA (1 µM)</th>
<th>Vector</th>
<th>mPLD2</th>
<th>mPLD2-mu.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Involvement of PLD2 in LPA-induced transactivation of platelet-derived growth factor receptor-beta in human bronchial epithelial cells

Lixin Wang, Rhett Cummings, Yutong Zhao, Andrius Kazlauskas, James Sham, Andrew Morris, Steve Georas, David N. Brindley and Viswanathan Natarajan

J. Biol. Chem. published online July 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302896200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts