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Lysophosphatidic Acid Signaling in Airway Epithelium: Role in Airway Inflammation and Remodeling

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Abstract

Lysophosphatidic acid (LPA), a potent bioactive phospholipid, induces diverse cellular responses, including cell proliferation, migration, and cytokine release. LPA can be generated intracellular and extracellularly through multiple synthetic pathways by action of various enzymes, such as phospholipase $A_{1/2}$ ($PLA_{1/2}$), phospholipase D (PLD), acylglycerol kinase (AGK), and lysophospholipase D (lysoPLD). Metabolism of LPA is regulated by a family of lipid phosphate phosphatases (LPPs). Significant amounts of LPA have been detected in various biological fluids, including serum, saliva, and bronchoalveolar lavage fluid (BALF). The most significant effects of LPA appear to be through activation of the G-protein-coupled receptors (GPCRs), termed $LPA_{1\text{-}6}$. LPA regulates gene expression through activation of several transcriptional factors, such as nuclear factor-κB (NF-κB), AP-1, and C/EBPβ. In addition to GPCRs, cross-talk between LPA receptors and receptor tyrosine kinases (RTKs) partly regulates LPA-induced intracellular signaling and cellular responses. Airway epithelial cells participate in innate immunity through the release of cytokines, chemokines, lipid mediators, other inflammatory mediators and an increase in barrier function in response to a variety of inhaled stimuli. Expression of LPA receptors have been demonstrated in airway epithelial cells. This review summarizes our recent observations of the role of LPA / LPA-Rs in regulation of airway epithelium, especially in relation to the secretion of pro- and anti-inflammatory mediators and regulation of airway barrier function.

Keywords

lysophosphatidic acid; airway epithelial cells; cytokine release; airway epithelium barrier function; G-protein-coupled receptors; RTKs; signal transduction; inflammation

1. Introduction

Phospholipids are major constituents of all biological membranes while lysophospholipids are present in relatively smaller proportion in tissues, biological fluids and cells. The prefix “Lyso” has been widely used by researchers in basic, translational and medical science to indicate loss or absence of a long chain fatty acid from the glycerol backbone of the glycerophospholipid. However, the prefix “Lyso” in the original context implied the ability of a phospholipid to induce lysis of cells, based on the observation that snake venom caused lysis of erythrocytes

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due to generation of a lipid from erythrocyte membranes, which was subsequently identified as lysophosphatidylcholine (LPC). Among the various lysoglycerophospholipids, lysophosphatidic acid (LPA) (1- or 2-radyl-*sn*-glycerol 3-phosphte) has the simple structure with either a long-chain saturated/monounsaturated fatty acid- or alkyl- or alk-1-enyl- moiety attached to *sn-1* carbon or a polyunsaturated fatty acid group linked to *sn-2*, and a phosphate group at *sn-3* position of the glycerol backbone. Similar to LPC, LPA is water soluble, present in nM to μM concentrations in plasma bound to either albumin or gelsolin, and plasma levels of LPA increase following activation of platelets and circulating monocytes/ polymorphonuclear leukocytes [1-7]. In addition to its role as an intermediate in *de novo* biosynthesis of phospholipids in mammalian tissues/cells, LPA functions as a serum-derived growth factor, and also exhibits multiple pleiotropic effects as an inter- and intra-cellular lipid mediator of cellular functions such as proliferation [8-14], migration [11,12,15-18], and survival [19-21]. Many of these cellular effects of LPA are mediated via specific G protein-coupled LPA receptors [22-30], which are present on the cell surface, intra-cellular organelles and the nucleus. Additionally, the peroxosome proliferator-activated receptor-γ (PPARγ) has been identified as an intracellular receptor for LPA [31,32]. LPA-Rs are coupled to multiple intracellular signaling pathways via heterotrimeric G_i, G_q, $G_{12/13}$, and G_s regulating cell proliferation, migration and survival [22-29,33-38]. While more than 60 reviews have dealt with the emerging role of LPA in proliferation, motility, and various diseases, there has been no mini- or comprehensive review that addresses the role of LPA in airway epithelium. Towes, M.L. et al. reviewed the effect of LPA on contraction, proliferation, and gene expression in airway smooth muscle cells in 2002 [39]. The present review focuses on LPA and its role in airway epithelial signaling, inflammatory responses, and remodeling with an emphasis on its pro- and anti-inflammatory effects in the airway.

2. Biosynthesis and catabolism of LPA

LPA is a natural constituent of all tissues, plasma [1-5,7], saliva [40], bronchoalveolar lavage fluid (BALF) [41-43], follicular fluid [44], malignant effusions [45], and mildly oxidized LDL [46]. Plasma levels of LPA are low (< 100 nM). However, serum concentrations of LPA are much higher (> 1000 nM) and partly derived from activated platelets [1,2,7]. Furthermore, the fatty acid composition of LPA derived from plasma is different compared to serum LPA, which has more polyunsaturated fatty acids [1,2,7]. Plasma levels of LPA are normally low and regulated by production, degradation, and uptake by tissues and circulating cells. Mechanisms that regulate low LPA levels in plasma under normal conditions as well as enhanced LPA production during injury/pathophysiology states are not well understood, although plasma contains the necessary enzymes and substrates for LPA production. LPA in biological fluids could arise from at least two sources. First, LPA can be synthesized in the cells and then released, or LPA can be synthesized outside of cells. *De novo* synthesis of LPA is regulated by two key enzymes, glycerophosphate acyl transferase [47,48] and acylglycerol kinase (AGK) [49,50], which are predominantly localized in microsomes and mitochondria, respectively. Glycerophosphate acyl transferase catalyzes the transfer of long-chain fatty acid from fatty acyl CoA to glycerol-3-phosphate to biosynthesize LPA, while acylglycerol kinase phosphorylates monoacylglycerol to form LPA.

2.1. Intracellular generation of LPA

At least two pathways have been identified for intracellular LPA generation. In the first pathway, phosphatidic acid (PA) generated by phosphorylation of diacylglycerol (DAG) catalyzed by DAG kinase or agonist-stimulated phospholipase D (PLD) signal transduction is converted to LPA, a process mediated by phospholipase (PL) A_1 or PLA_2 type enzymes [7, 51-54]. While the specificity of PLA_1 or PLA_2 in using PA as a substrate *in vivo* is unclear, two membrane-bound PA-specific $mPLA_1$ α and $mPLA_2$ β, also called LIPH and LIPI

belonging to the lipase gene family [54,55] has been demonstrated, which may have specific role(s) in generating polyunsaturated 2-acyl-LPA. Interestingly, both $mPLA_1$ α and $mPLA_2$ β are located in lipid rafts indicating the possibility of LPA generation in the raft microdomains [54,55]. However, the physiological implication of this distribution in lipid microdomain remains to be established. Although the role of the PLD/PA pathway in generation of LPA in airway cells has not been demonstrated, incubation of SK-OV-3 cells (ovarian cell line) with 1-butanol, which diverts PA formed by PLD to phosphatidylbutanol, caused a consistent reduction of 50 % of constitutively produced LPA and ~ 60 % of inducible LPA production by LPA treatment [51]. Further, Luquain et al. showed that overexpression of PLD2 (but not PLD1) resulted in LPA production by ovarian cancer cells in response to agonists stimulation [51]. The second pathway of intracellular generation of LPA is mediated by AGK, an enzyme that phosphorylates monoacylglycerol (MAG) to LPA [47-50] wherein MAG is derived either by the action of lipid phosphate phosphatases (LPPs) on LPA or lipase(s) on DAG [56-58]. In human bronchial epithelial cells (HBEpCs), overexpressed lentiviral V5-tagged AGK co-localized with MitoTracker Red [60], which was similar to mitochondrial localization of AGK in NIH 3T3 fibroblasts, HEK 293, and PC-3 cells [49,50]. Furthermore, cell lysates of over-expressing V5-tagged AGK phosphorylated MAG and DAG, but not sphingosine or ceramide [60]; however, AGK expressed in bacteria phosphorylated MAG and DAG as well as sphingoid bases [49,59]. AGK expression was up-regulated in prostate cancers compared to normal prostate tissues, and expression of AGK in PC-3 prostate cancer cells increased formation and secretion of LPA [50]. However, in HBEpCs, over-expression of AGK increased intracellular production of LPA, but the intracellularly generated LPA was not secreted [60], suggesting differences between the two cell types in the mechanism of action of intracellularly generated LPA signal transduction (Figure 1).

2.2. Extracellular LPA generation

In this pathway, secretory PLA_2 ($sPLA_2$), phosphatidylserine (PS)-specific PLA_1, and lysoPLD [also named as ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) or autotaxin (ATX)] contribute to extracellular production of LPA. In activated platelets and other cell types, LPC, lysophsophatidylethanolamine (LPE), and lysophosphatidylserine (LPS) are generated by $sPLA_2$ and PS-specific PLA_1 in plasma [61,62]. LPC is also formed from PC in lipoproteins by lecithin: cholesterol acyltransferase (LCAT) and PLA_1 type enzymes [63,64]. Lysophospholipids, thus generated, are acted upon by lysoPLD or autotaxin to LPA. LysoPLD activity, originally described in rat plasma [65], converts exogenously added LPC to LPA. Subsequent purification and characterization of lysoPLD from plasma showed that this was identical to autotaxin (ATX) [6]. ATX, in addition to generation of LPA from lysophospholipids, also produces cyclic phosphatidic acid (cPA), which contains a dioxaphospholane ring at *sn-2* and *sn-3* positions of the glycerol backbone [66-69]. cPA is also present in human serum [69-71] and its biological activities are distinct from that of LPA, exhibiting anti-proliferative and anti-metastatic properties *in vivo* and *in vitro* [66-68,72]. In addition to hydrolysis of LPA, lysoPLD/ATX also possesses ecto-nucleotide pyrophosphatase/phosphodiesterase activity [73,74]. Overexpression of lysoPLD mRNA in non-small cell lung cancer has been reported [75], however, the role of lysoPLD/autotaxin in airways and airway diseases is unknown. ATX deficiency is embryonically lethal and ATX (-/-) mice die at embryonic day 9.5 with profound vascular defects [76,77]. Furthermore, $sPLA_2$ activity was increased in BALF after inhaled antigen challenge in asthmatics [78,79], and late-phase allergic reactions were characterized by increased phospholipids and lysophospholipids in BALF [80,81].

2.3. LPA degradation

Three major pathways have been described for LPA degradation in mammalian systems. The first pathway involves dephosphorylation of LPA to MAG by phosphatases that belong to

phosphatidate phosphatases type 2 (PAP-2), also known as lipid phosphate phosphohydrolase (LPP). There are three major isoforms of LPPs, LPP-1-3, that have been cloned and characterized in mammals [82-85]. While all the isoforms dephosphorylated, *in vitro*, a variety of lipid phosphates including LPA, PA, S1P, and ceramide-1-phosphate, LPA is a preferred substrate for LPP-1 compared to LPP-2 and LPP-3 [85]. Expression of LPP-1-3 was demonstrated in human bronchial epithelial cells (HBEpCs) by real time RT-PCR and Western blotting with LPP-specific antibodies [58]. Exogenous addition of [^{3}H]oleoyl LPA was hydrolyzed to [^{3}H]MAG, while over-expression of LPP-1 Wt enhanced LPA hydrolysis by ~2-3 fold compared to vector infected control cells [58]. As LPP-1 is an ecto-enzyme, one of its roles is to modulate LPA levels in milieu, which regulates LPA signaling and cell functions. The second pathway of LPA degradation involves conversion of LPA to PA catalyzed by a LPA acyltransferase (LPAAT) [86-90], which has not been well characterized in the airway. The third pathway responsible for LPA degradation involves hydrolysis of the long-chain fatty acyl group from LPA by the action of lysophospholipases. At least two distinct lysophospholipases with specificity towards LPC and LPA have been characterized [91-94]. The participation of lysophospholipases in LPA clearance from BALF and alveoli is unclear.

2.4. LPA levels in BALF

LPA is present in human BAL fluids as determined by tandem mass spectrometry (LC-MS/MS), and significantly increased following segmental allergen challenge [41]. Interestingly, analysis of the LPA by tandem MS/MS revealed that 20:4, 22:4, and 22:6 LPA molecular species were increased following segmental allergen challenge [41]. These increased accumulations of polyunsaturated LPA species, generated from complex phospholipids by activated phospholipases, are consistent with catabolic LPA production during lower airway inflammation. However, a role for lysoPLD/autotaxin in enhanced LPA production in the BALF after allergen challenge has not been established. In murine models of asthma, *Schistosoma mansoni egg/soluble antigen* or ovalbulmin challenge increased LPA levels in BALF by ~2-3 fold compared to control mice [43]. Recent studies by Tager et al. showed that LPA levels were increased in BALF in a murine model of bleomycin-induced pulmonary fibrosis, and in BALF from idiopathic pulmonary fibrosis (IPF) patients, compared to normal controls [42]. Furthermore, inhibition of LPA_1 attenuated fibroblast chemotaxis induced by IPF BALF samples [42]. These studies from segmental allergen challenged patients [41] and IPF patients [42] show that elevated LPA levels in BALF may be a biomarker of airway inflammatory diseases. Further investigations on the mechanism(s) and cell type(s) involved in LPA production in normal and pathological conditions are necessary to understand the physiological role of LPA in airway inflammation.

3. LPA receptors

3.1. LPA receptors and G proteins

Discovery of LPA specific receptors in the plasma membrane of mammalian cells has led researchers to investigate LPA-Rs in LPA-induced intracellular signaling, and biological/physiological/pathological roles. Chun et al., for the first time, showed that LPA was a ligand for ventricular zone-1 (vzg-1) receptor, which is a member of the endothelial differentiation gene (EDG) family [23]. To date, six cell-surface LPA receptors, LPA_{1-6}, have been cloned and described in mammals [22-29,38,95]. Based on sequence homology, three of these receptors belong to the EDG subfamily of the G-protein-coupled receptor (GPCR) superfamily. LPA_1/EDG2, LPA_2/EDG4, and LPA_3/EDG7 share ~50% sequence homology [33-35,95], while LPA_4 /GPR23/P2Y_9, and LPA_5/GPR92, and LPA_6/GPR87 are structurally distinct from the EDG family and share < 40% homology with conventional LPA_{1-3} [26-29]. The biological effects of LPA are mediated by ligation to specific LPA-Rs that are coupled to heterotrimeric G-protein families, the G_s, G_i, G_q, and $G_{12/13}$. LPA_1 and LPA_2 are known to interact with

G_i ,G_q, and $G_{12/13}$; LPA_3 interacts with G_i and G_q but not $G_{12/13}$ [33,36]; LPA_4 appears to couple with all the G proteins [26,37], and LPA_5/GPR92 is likely coupled to G_s, $G_{12/13}$, and G_q [27]. LPA signaling via LPA-Rs include: 1) the activation of phospholipase C (PLC) and calcium mobilization mediated by G_q [96-99]; 2) G_i-mediated inhibition of adenylate cyclase [100,101], activation of the Ras-MAPK cascade [9,15], and activation of PI3K-PKB/Akt signaling [10,102], which promote cell motility and suppresses apoptosis; 3) $G_{12/13}$-mediated activation of Rho/Rac GTPases signaling, which regulates cytoskeleton rearrangement [11, 12,103]; and 4) G_{13}-mediated membrane depolarization of chloride channels [5,104]. LPA_4 and LPA_5 activate adenylate cyclase, resulting in the accumulation of cAMP and intracellular [Ca^{2+}] [26,27]. LPA also activates Src [105-107], PYK2 [105,108], PKC [115-120] and PLD [115-120], transactivates growth factor receptors (EGF-R and PDGF-Rβ) [115,120-129], induces COX-2 expression and PGE_2 secretion [115,127,130-133], and regulates transcriptional factors such as C/EBPβ [115,132], NF-κB [58,114,115,132,134,135] and AP-1 [115,132,135-137].

3.2. LPA receptors in airway epithelium

The mRNA expression profile of LPA receptors in HBEpCs [41,120,135,139], nasal polyp-derived epithelial cells [138], and airway epithelial cell lines [138] has been described. In HBEpCs, the relative abundance of mRNA detected was $LPA_1 > LPA_3 > LPA_2 > LPA_4$, while LPA-$R_5$ was not detectable in HBEpCs [139]. LPA_{1-3} are not only localized at the plasma membrane, but are also distributed in the cytoplasm of the cells [120]. Studies from Georas et al. showed that LPA_1 and LPA_2 are expressed in airway epithelial cells of human lung tissue [41]. Furthermore, the expression of LPA_1 on HBEpCs cell surface was determined by flow cytometry with an LPA_1 receptor antibody directed against the conserved extracellular N-terminus [41]. The expression of LPA_1 is not restricted to the plasma membrane and cytoplasmic organelles as nuclear localization of LPA_1 has been shown in various cell types, including HBEpCs [30]; however, the biological role of nuclear LPA_1 in airway epithelium remains to be investigated. In contrast to primary HBEpCs, only LPA_1 and LPA_2, but not LPA_3 or LPA_4, were expressed in epithelial cell lines A549 and BEAS-2B [138]. Nasal polyp epithelial cells exhibited expression of the LPA_1, LPA_2 and LPA_4, but not LPA_3 [138], suggesting differences in expression profile in different epithelial cell types. The regulation of LPA receptor expression is not well studied. The expression of LPA receptors are modulated differently in asthmatic HBEpCs compared to normal controls. In asthmatic HBEpCs, LPA_1 mRNA was up-regulated ~ 3.2 fold, while LPA_2 was down-regulated ~ 2.6 fold compared to normal HBEpCs [139]. Both IL-13 and IFN-γ significantly reduced LPA_1 and LPA_2 mRNA levels, while IL-4 had no effect on LPA receptor mRNA expression in the A549 epithelial cell line [138]. However, the effect of IL-13 and IFN-γ on LPA-induced signaling and biological responses remains to be investigated.

3.3 LPA receptors null mice in pulmonary/airway diseases

Given that LPA levels are elevated in BALF from segmental allergen challenged asthmatics [41], a bleomycin model of pulmonary fibrosis [42], and a Schistosoma mansoni egg/soluble antigen challenge murine model of inflammation [43], it becomes critical to determine the role of LPA/LPA-Rs underlying the pathobiology of airway/pulmonary diseases. Genetically engineered LPA_{1-3} null mice have been used to study a number of pathophysiological states including airway inflammation, asthma, and pulmonary fibrosis. LPA_1 (+/-) mice or LPA_2 (+/-) mice sensitized with inactivated *Schistosoma mansoni eggs and challenged with soluble antigen Schistosoma mansoni eggs* showed less infiltration of eosinophils, decreased PGE2 levels in BAL fluids, and lower COX-2 expression in the lung tissue [43]. Both $LPA_{1\&2}$ seem to be involved in the *Schistosoma mansoni egg/soluble antigen* challenge murine model of inflammation. Furthermore, 6 h after intratracheal administration of LPA (5 μM) to $LPA_{1\&2}$ (+/-) mice resulted in less infiltration of neutrophils into alveolar space compared to LPA-

challenged Wild type mice [43]. LPA_1 (-/-) mice showed significant increase in CD3/CD28-driven proliferation after ovalbumin challenge [140], suggesting an anti-inflammatory role for LPA_1 in asthma. In the bleomycin model of lung injury mediated by fibroblast recruitment and vascular leak, mice lacking LPA_1 were markedly protected from fibrosis and mortality suggesting a link between LPA_1 and development of pulmonary fibrosis [42]. These studies indicate a potential role of LPA-Rs in lung injury, inflammation, and pulmonary fibrosis; however, the relative contribution of LPA-Rs present on the epithelial cells vs. endothelial vs. infiltrating cells to the inflammation or injury process is unclear that might require tissue targeted null knockdown of LPA-Rs.

3.4 LPA signaling via LPA-Rs in the airway epithelial cells

The role of LPA receptors in LPA signaling in HBEpCs has been investigated in the context of cytokine/chemokines secretion. Down-regulation of LPA_1, or LPA_2, or LPA_3 by specific siRNA attenuated LPA-induced IL-8 secretion in HBEpCs, and LPA_3 specific agonist increased IL-8 secretion in HBEpCs [135]. LPA signals via LPA receptors through different heterotrimeric G proteins, such as G_i, G_q, G_s, and $G_{12/13}$, which couple to the C-terminus domain of LPA receptors. Pertussis toxin (PTx), which uncouples GPCR from its $G_{i/o}$ and in turn reduces the affinity of GPCR toward its agonist, serves as a tool to study G_i-mediated signaling. In HBEpCs, PTx attenuated LPA-induced phosphorylation of EGF-R [128] and c-Met [113], as well as expression of IL-8 [114], COX-2 [115], and IL-13Rα2 [136]. To further investigate the involvement of G-proteins in LPA signaling, Cummings et al. transfected HBEpCs with minigenes that encode peptides that specifically block the respective G-protein/receptor interface [114]. Expression of $G_{12/13}$ or G_i blocking peptides, but not G_q blocking peptide, resulted in attenuation of LPA-induced IL-8 secretion [114]. These studies suggest that LPA induced signaling in HBEpCs is through G_i and $G_{12/13}$-coupled LPA receptors.

4. LPA regulates expression of pro- and anti-inflammatory genes

Airway epithelium is the first point of contact for inhaled substances such as cigarette smoke, microorganisms, allergens and environmental pollutants, and plays a key role in host defense and innate immune response [141-144]. The involvement of the airway epithelium in modulating immune responses is regulated by secretion of cytokines and other mediators of the airway epithelium in response to various stimuli. The earlier observations of LPA as the serum factor mediating serum-induced sensitization of cAMP responses in airway smooth muscle cells [39,145], and a potential role of LPA in asthma [41,43,140] and IPF [42] have resulted in several investigations related to LPA in innate immunity, inflammation, repair and remodeling and gene expression in airway cells and animal models of airway diseases.

4.1. LPA modulates expression and release of cytokines and lipid mediators

4.1.1. IL-8 expression and secretion—IL-8, a major chemoattractant of neutrophils, plays a key role in innate immune responses [114,146,147]. Higher levels of IL-8 have been found in BALF of patients of airway inflammatory diseases including acute lung injury [114, 119]. LPA has been shown to be a strong stimulus for IL-8 secretion in various airway epithelial cell types, including HBEpCs, BEAS-2B, and A549 [58,114,128,138,139]. In HBEpCs, LPA mediated IL-8 gene expression at 1 h, and peaked at 2h, while IL-8 secretion by LPA reached its peak at 12 h [114]. LPA-induced IL-8 secretion was through G_i and $G_{12/13}$-coupled LPA_{1-3} receptors [114,135]. A similar increase in IL-6 and IL-8 production after LPA stimulation was observed in BEAS-2B cells [138]. Consistent with the *in vitro* studies, intratracheal administration of LPA in mice increased MIP-2 (the murine homolog of IL-8) levels at 3 h and neutrophils infiltration at 6 h. At later periods (>12 h), the levels of MIP-2 and neutrophils returned to near basal levels [114]. Hashimoto et al. also showed that inhalation induced histamine release and increased numbers of eosinophils and neutrophils in BAL of

guinea pig [148,149]. These results suggest that LPA regulates airway inflammation via stimulating cytokine and inflammatory mediator release and neutrophils or eosinophils infiltration in airway.

4.1.2. IL-13R alpha2 expression and release—The levels of interleukin-13 (IL-13), a Th2-type cytokine, are increased in BALF of asthma patients [150,151] and ovalbumin challenged mice [152]. IL-13 induces phosphorylation of signal transducer and activator of transcription 6 (STAT6), which activates the transcription of many pro-inflammatory genes in epithelial cells and plays a critical role in the pathogenesis of bronchial asthma [153-155]. IL-13Rα1 binds to IL-13 and mediates IL-13-induced activation of Janus kinases (JAKs) and STAT6 [156,157]. The human IL-13 decoy receptor, termed IL-13 Receptor α2 (IL-13Rα2), binds to IL-13 with much higher affinity (Kd=0.25-1.2 nM) compared to IL-13Rα1 (Kd=2-10 nM) [158-160], and overexpression of IL-13Rα2 selectively inhibited IL-13-induced response in airway epithelium and murine lung [161-163]. Elevated IL-13Rα2 mRNA expression has been detected in *Schistosoma* egg-induced liver fibrosis [164] and bleomycin-induced fibrosis [165]. In addition, interferon-γ (IFN-γ) or IL-13 treatment induced IL-13Rα2 mRNA expression that was localized to the cell surface in U937 [35] and bronchial epithelial cells [167]. While LPA induced IL-13 expression under conditions of semimaximal activation in T cells, in HBEpCs [168], LPA treatment induced IL-13Rα2 mRNA and protein expression and secretion through G_i-coupled LPA receptors, without altering IL-13Rα1 expression [136]. Further, elevated IL-13Rα2 expression by LPA in HBEpCs attenuated IL-13-mediated phosphorylation of STAT6 and eotaxin and SOCS-1 gene expression [136]. As LPA levels are elevated in BALF from segmental allergen challenged asthmatics [41] and IPF [42], and LPA modulates expression of IL-13Rα2 and IL-13-mediated STAT6 signaling [136], it is postulated that increased LPA levels may have a protective role in airway inflammation and remodeling of airways. Future studies related to LPA and its effect on production of Th2 cytokines, and airway remodeling and repair would increase our understanding of how LPA and its analogs ameliorate airway inflammation in asthma and fibrosis.

4.1.3. COX-2 expression and PGE2 release—Prostaglandin E_2 (PGE_2) is an autocrine lipid mediator derived from arachidonic acid (AA) metabolism by cyclooxygenase-1 or 2 (COX-1 or 2) [169]. PGE_2 plays crucial roles in various biological events such as neuronal function [170], vascular hypertension [171], tumorigenesis [172-174], and inflammation [175]. Upregulation of COX-2 expression and PGE_2 release plays a protective role in the innate immunity response and attenuated allergen-induced tissue repair process in airway inflammation [176,177]. Inhaled PGE_2 airway responses, hyperresponsiveness, and inflammation [178,179]; however, the regulation of COX-2 expression and the physiological effect of PGE_2 on airway epithelium has not been well examined. LPA challenge increased COX-2 expression and PGE2 production in HBEpCs [115]. Analyses of total RNA by Real-time RT-PCR showed that both COX-1 and COX-2 were expressed in HBEpCs and that the expression of COX-1 is ~ 2 fold higher than COX-2 [115]. Exposure of HBEpCs to LPA (1 μM) induced COX-2 gene and protein expression and PGE_2, while such exposure had no effect on COX-1 gene and protein expression up to 24 h [115]. Further, PTx attenuated LPA-induced COX-2 mRNA and protein expression, suggesting the involvement of G_i-coupled LPA-Rs. COX-2 siRNA effectively down-regulated COX-2 protein expression and attenuated LPA-induced PGE_2 release by ~ 50 % compared to scrambled siRNA cells exposed to LPA [115]. These results demonstrated that LPA induced PGE_2 secretion was dependent on expression of COX-2 in HBEpCs. As PGE_2 exhibits anti-inflammatory properties in the airway [176-179], enhanced COX-2 expression and PGE_2 release induced by LPA may have a protective role in airway inflammation and remodeling.

4.2. LPA regulates transcriptional factors

4.2.1. Nuclear factor-κB (NF-κB)—NF-κB and AP-1 are transcriptional factors expressed in almost all mammalian cell types and play a key role in regulating the immune response to infection. NF-κB is a heterodimer composed of two Rel family members (RelA and B). In quiescent cells, NF-κB is present in cytosol in an inactive form bound to an inhibitory protein, I-κB, and after stimulation, I-κB is phosphorylated, and degraded by the 26S proteasome. NF-κB is released from NF-κB/I-κB complex, translocates from cytosol to nucleus, and activates gene transcription [180]. The activation of NF-κB by LPA in HBEpCs has been detected by several procedures including phosphorylation of I-κB, NF-κB nuclear translocation, NF-κB luciferase reporter assay, and electrophoretic motility-shift assay [58,114,115,135]. Signaling pathways regulating LPA-induced NF-κB activation in HBEpCs have been partially defined. LPA induced NF-κB activation via p38 MAPK [139] and protein kinase C δ (PKCδ) [114]. The promoter region of human IL-8 and COX-2 have potential NF-κB binding elements [181,182], however, the human IL-13Rα2 promoter region lacks NF-κB binding elements [183]. Inhibition of NF-κB activation attenuated LPA-induced IL-8 [114] and COX-2 [115] expression, suggesting that NF-κB plays a critical role in LPA-induced gene expression in HBEpCs. However, LPA-induced IL-13Rα2 expression was not dependent on the NF-κB pathway in HBEpCs [136].

4.2.2. C/EBPβ—The C/EBP family consists of six proteins that belong to the basic zipper transcriptional factors [184]. The C/EBPβ is important in the regulation of genes involved in immune and inflammatory responses and has been shown to bind to transcriptional regulatory regions of several acute-phase and cytokine genes. The human COX-2 promoter region has been sequenced and contains a putative transcriptional regulatory element of C/EBPβ [182]. The participation of C/EBPβ in COX-2 expression to various stimuli in different cell types has been described [185,186]. In HBEpCs, LPA induced phosphorylation of C/EBPβ and downregulation of C/EBPβ expression by siRNA attenuated LPA-induced COX-2 expression [115], suggesting the involvement of C/EBPβ in LPA-induced gene expression in HBEpCs. Further evidence showed that LPA-induced activation of PLD2, PKCζ, and EGF-R contributed to C/EBP activation in HBEpCs [115]. The role of other C/EBP family members in LPA-mediated cytokine and lipid mediators release is still not clear.

4.2.3. JNK and p38 MAPK in LPA-induced NF-κB and AP-1 Activation—c-Jun N-terminal kinase (JNK) and p38 MAPK belong to MAP kinase family, which are important component of signal transduction pathways induced by growth factors, cytokines, and GPCR ligands. p38 MAPK and JNK1 and JNK2 isoforms are widely distributed in mammalian cells [187]. LPA treatment of HBEpCs induced phosphorylation of JNK1/2 [115,135,136] and c-Jun [135], induced c-Jun nuclear localization [135], and induced AP-1 activation [135]. The mechanism(s) of regulation of JNK activation by LPA is still not clear. Interestingly, overexpression of catalytic dominant negative PLD1 and 2 isoforms attenuated LPA-induced phosphorylation of JNK in HBEpCs [136], suggesting JNK activation may be regulated by PLD-generated phosphatidic acid (PA). Inhibition of JNK activation by JNK inhibitor (JNKiII) or downregulation of c-Jun by c-Jun siRNA attenuated LPA-induced activation of AP-1 [135], as well as IL-8 [135], COX-2 [115], and IL-13Rα2 [136] expression.

LPA-induced activation of NF-κB has been shown to be dependent on activation of p38 MAPK. An inhibitor of p38MAPK or p38 MAPK siRNA blocked phosphorylation of I-κB and NF-κB translocation to the nucleus without affecting JNK/ c-Jun phosphorylation and AP-1activation [135]. The role of LPA-induced activation of p38 MAPK in LPA-mediated IL-8 and COX-2 expression was investigated in HBEpCs. Inhibition of p38 MAPK activation attenuated LPA-induced IL-8 [135] through decreasing the NF-κB pathway, not the JNK/AP-1 pathway. Recent studies suggest that p38 MAPK also regulates COX-2 mRNA stability

[188]. In HBEpCs, LPA induced COX-2 mRNA expression as well as COX-2 mRNA stability [189], while downregulation of p38 MAPK activation attenuated LPA-induced COX-2 mRNA stability and expression [189]. p38 MAPK regulates LPA-induced COX-2 expression through both transcriptional and post-transcriptional regulation. Further study will determine the role of p38 MAPK isoforms in the regulation of COX-2 mRNA levels in HBEpCs.

4.3. LPA activates PKC isoforms

PKC is a superfamily of kinases that phosphorylates protein substrates on serine and threonine residues and transduces the cellular signals. Eleven PKC isoforms have been identified in mammals and divided into three subgroups: classical PKCs (cPKCα, βI, βII, and γ), novel PKCs (nPKCδ, ε, η, μ and θ), and atypical PKCs (aPKCζ and γ) based on sequence homology. The cPKCs are calcium-dependent, and activated by diacylglycerol (DAG) and DAG-mimicking phorbol esters. The nPKCs are calcium-independent, activated by DAG and DAG-mimicking phorbol esters. The aPKCs are structurally divergent from other PKC family members and activated by acidic phospholipids such as phosphatidylserine (PS), polyphosphoinositides (PI) and phosphatidic acid (PA), but not by DAG or phorbol esters [190]. Expression of PKCα, δ, ι/λ, and ζ in HBEpCs were confirmed by Western blotting with specific antibodies to these isoforms [114]. LPA induced phosphorylation and plasma membrane translocation of PKCδ [114] and ζ [189]. The involvements of PKC isoforms in IL-8, IL-13Rα2, and COX-2 expression have been studied by using PKC isoform specific pharmaceutical inhibitors or overexpression of dominant negative PKC isoforms (dn-PKCs) in HBEpCs. PKCδ contributes to LPA-induced IL-8 [114], IL-13Rα2 [136], and COX-2 [189] expression. PKCζ regulates LPA-induced COX-2 expression [115] while PKCλ regulates LPA-induced IL-8 expression [114] in HBEpCs. It has been shown that PKC inhibitors attenuated activation of transcriptional factors in various cell types [191,192]. Inhibition of PKCδ attenuated LPA-induced NF-κB activation [114] and inhibition of PKCζ modulated LPA-induced C/EBPβ activation in HBEpCs [115]. Regulation of PKCζ by PLD has been shown in human pulmonary arterial endothelial cells (HPAECs) [193] while the relationship between PKCζ and PLD will need to be further determined in human airway epithelial cells (Fig. 2).

4.4. Role of LPPs in regulation of LPA signaling

Recent studies have revealed that LPA degradation occurs through the LPPs family, which consists of three family members (LPP-1, LPP-2, LPP-3), attenuated LPA-induced intracellular signaling [82-85]. LPPs contain a novel conserved phosphatase sequence motif, $K(X)_6RP$-$(X_{12\text{-}54})$-PSGH-$(X_{31\text{-}54})$-$SR(X)_5H(X)_3D$, that lies on the outside of the cell, or on the luminal surface of the endoplamic reticulum or Golgi [57,85]. In the rat, the activity of LPPs in the lung is the highest among any tissue and mRNA expression for LPP-1 and LPP-3, but not LPP-2, was detected in rat lung [194]. LPPs activity is enriched in lipid-rich signaling platforms isolated from type II cell-mouse lung epithelial cell lines (MLE12 and MLE15) [195,196], and using Western blotting and RT-PCR, expression of LPP-1, -2, and -3 were detected in HBEpCs [58]. In HBEpCs, exogenously added LPA was hydrolyzed by a LPP-like ecto-enzyme activity, which was inhibited by tyrosine kinase inhibitors, but not by serine-threonine inhibitors. Overexpression of LPP-1 Wild type (Wt) decreased extracellular LPA levels and attenuated LPA-induced intracellular Ca^{2+}, ERK1/2 phosphorylation, activation of NF-κB pathway, secretion of IL-8, and expression of COX-2 [58,189]. Downregulation of LPP-1 by LPP-1 siRNA enhanced LPA-induced COX-2 expression in HBEpCs [189]. These results suggest that LPP-1 regulated LPA-induced gene expression via degradation of extracellular LPA. Interestingly, overexpression of LPP-1 also attenuated the IL-8 secretion mediated by a non-hydrolyzed LPA analogue, suggesting an intracellular role of LPP-1 in regulation of LPA-induced signaling [58]. As LPA plays a critical role in innate immune response in airway

diseases, and LPP-1 attenuates LPA function by its ecto-enzyme activity, targeting LPP-1 may regulate airway inflammation.

5. Cross-talk between LPA-Rs and Receptor Tyrosine Kinases (RTKs)

Several lines of investigation in a variety of cell types have shown that many of the growth promoting effects of GPCR agonists (LPA, sphingosine-1-phosphate (S1P), angiotensin, PGE_2, and others) are mediated through activation of receptor tyrosine kinases (RTKs), a phenomenon termed as "Transactivation" [197-199]. The transactivation of RTKs by GPCR ligands has been demonstrated in various cell types and this cross-talk between GPCRs and RTKs provides additional non-canonical signaling pathways, which regulate cellular function. In addition, disruption of GPCR function via PTx or antagonist reduces the growth factor stimulated activation of ERK [200]. Both the GPCR ($S1P_1$) and the βγ subunit of the activated G-protein form a functional signaling complex with the RTK, which may reprogram the mitogenic signal of the RTK to a migratory response [200]. Thus, potential signaling via "Transactivation" between GPCRs and RTKs will allow spatial regulation of effectors dictating nature of the biological responses.

5.1. LPA transactivates PDGF-Rβ

Among the various RTKs, GPCR mediated transactivation of platelet-derived growth factor receptor (PDGF-R) has been well described [198,200-202]. The PDGF has been recognized as an important growth factor regulating cell proliferation and the development of many diseases including cancer [203-205]. PDGF-BB challenge stimulated phosphorylation of Erk1/2, suggesting that PDGF plays a proliferative role in HBEpCs [120]. In addition to PDGF-BB, LPA also induced tyrosine phosphorylation of PDGF-Rβ via a transactivation mechanism involving PLD2, but not PLD1, signaling [120]. Furthermore, transactivation of PDGF-Rβ by LPA regulated LPA-dependent phosphorylation of Erk1/2 [120] in HBEpCs. These results suggest that cross-talk between LPA-Rs and PDGF-Rβ may play a role in airway remodeling and repair. While the LPA_1-PDGF-Rβ signaling complex could not be identified in HBEpCs [212], interaction between G-protein-coupled S1P1 and PDGF-Rβ has been identified and extensively studied in HEK293 and airway smooth muscle cells [201]. The formation of this signaling complex between $S1P_1$ and PDGF-Rβ permits the usage of βγ-subunits of activated G_i by PDGF-R to enhance PDGF signaling [206]. The functional implications of the "Transactivation" between RTKs and GPCRs in health and disease warrant more investigation.

5.2. LPA transactivates EGF-R

EGF-R, similar to other growth factor receptors, is activated by EGF, heparin binding-EGF (HB-EGF) and tumor growth factor-α (TGF-α) [207] b Since the first report of transactivation of EGF-R by LPA in fibroblast [208], recent studies have established EGF-R transactivation by LPA in airway epithelial cells [123,124,128,209,210]. Two pathways of LPA-mediated transactivation of EGF-R have been described: the first involves activation of matrix metalloproteinases (MMPs), cleavage of pro-HB-EGF, and subsequent ligation and activation of EGF-R [125,128,209,211], and the second pathway is HB-EGF shedding-independent involving the intracellular activation of EGF-R by Src kinases [209,210]. In HBEpCs, LPA treatment induced tyrosine phosphorylation of EGF-R at different tyrosine residues, which were Gi-dependent [128,212]. Furthermore, the LPA-induced EGF-R transactivation was regulated by PKCζ [115] and δ and Src kinases [128]. Among Src kinase family, using specific siRNA to down-regulate Src kinases, a role for Lyn kinase, but not Src or Yes kinase, in regulating LPA-induced EGF-R transactivation was established [128]. Similar to the mechanism of PDGF-Rβ transctivation by LPA, PLD2, but not PLD1, regulated the LPA-induced phosphorylation of EGF-R in HBEpCs [115]. Interestingly, LPA-mediated activation of Lyn was dependent on PKCδ signal transduction, with subsequent activation of MMP-2/9,

and proHB-EGF shedding [128]. LPA induced PKCζ activation through PLD2 [115]. It is not clear if PKCδ is up-stream or down-stream of PLD2 in HBEpCs. Data on the physiological significance of EGF-R transactivation by LPA is limited. Inhibition of EGF-R tyrosine kinase with AG1478 attenuated LPA-induced phosphorylation of ERK 1/2 in PC12 [213], ovarian cancer cells [214], prostate cancer cells [50], and HBEpCs [212], suggesting a potential role in cell proliferation. Furthermore, He et al. demonstrated that though all NF-κB, AP-1, and C/EBPβ are involved in LPA-induced COX-2 expression and PGE_2 release, however, only C/EBPβ is downstream signal molecule to EGF-R transactivation in HBEpCs [115] (Fig. 3). At present, it is unclear which of the LPA-Rs are involved in LPA-induced EGF-R transactivation. Another consequence of LPA-induced transactivation of EGF-R is a rapid and sustained decrease in EGF-R binding in human primary airway epithelial cells and BEAS-2B cell line [123,124]. The rapid decrease in EGF-R binding via EGF-R transactivation mediated by LPA was dependent on MEK/ERK and PKC, whereas the sustained decrease was primarily regulated by the PKC signaling pathway in BEAS-2B cell line [123]. Further studies are necessary to delineate physiological significance(s) of EGF-R transactivation in airway inflammation, repair and remodeling.

5.3. LPA trans*in*activates c-Met

c-Met, a proto-oncogene product, is a RTK, the receptor for hepatocyte growth factor (HGF), and predominantly expressed in various types of epithelium [215-216]. Increased expression and activation of c-Met is associated with tumor cell growth, scattering, invasion and metastasis [216-217]. Specific mutations of the tyrosine residues in the juxtamembrane and semaphorin domains of c-Met resulted in the regulation of the cytoskeleton through the focal adhesion protein paxillin [218]. LPA or S1P mediation enhanced tyrosine phosphorylation of c-Met via a transactivation pathway in human colon cancer cell lines and human gastric cancer cells [127,219]; however, in HBEpCs, unlike LPA-mediated tyrosine phosphorylation of PDGF-Rβ and EGF-R [115,120,128], LPA-induced serine, but not tyrosine, phosphorylation, which was dependent on activation of PKCδ by LPA [113]. Further, serine phosphorylation of c-Met by LPA attenuated HGF-mediated activation of AKT and cell motility in HBEpCs, suggesting a regulatory role for c-Met serine phosphorylation in epithelial cell motility [113]. These results suggest that in the same type of cells, the same GPCR ligand treatment might induce “yin and yang” responses on different RTKs (Fig. 4). Recent study has demonstrated that the HGF-dependent tyrosine phosphorylation of c-Met was largely suppressed by PKCδ with a reciprocal relationship to Ser-985 phosphorylation in A549 cell line [220]. In HBEpCs, LPA induced c-Met relocalization from the cytoplasm to cell-cell contacts and this was dependent on activation of PKCδ and ζ and interaction with E-cadherin, suggesting a role of c-Met in cell adhesion and motility [113]. The role of c-Met in regulation of airway epithelium barrier function and cytokine release will be investigated.

6. LPA regulates epithelial barrier via adherens junction proteins

The epithelial cell-cell junctional complex is composed of tight junctions, adherens junctions and desmosomes [221,222]. These adherens junctions play a pivotal role in regulating the activity of the entire junctional complex since the formation of adherens junctions subsequently leads to the formation of other cell-cell junctions, including tight junction [221-223]. The major adhesion molecules in the adherens junctions are the cadherins [221-223]. E-cadherin is a member of the cadherin family that mediates calcium-dependent cell-cell adhesion [221-225]. The N-terminal ectodomain of E-cadherin contains homophilic-binding domain, and the cytoplasmic domain binds to catenins, which interact with actin [221-225]. The regulated expression and the plasma membrane localization of E-cadherin are critical for the maintenance of epithelial cell-cell junctions crucial to the functional integrity of the epithelial barrier [221-223]. The decrease of adhesive properties of E-cadherin has been shown to be

related to the loss of differentiation and the subsequent acquisition of a higher motility and invasiveness of epithelial cells [226-228]. Dislocation or shedding of E-cadherin in the airway epithelium induces epithelial shedding and increases airway permeability in airway diseases [229-231]. However, the regulation and mechanism(s) of E-cadherin localization within the epithelium is not fully known, especially during pathological situations of inflammation and airway remodeling. LPA treatment tightened the airway epithelial cell barrier [41,232]; however, the mechanism(s) regulating LPA-induced epithelial barrier function is yet to be fully defined. In HBEpCs, exposure to LPA enhanced accumulation of E-cadherin and c-Met complex at cell-cell contacts through activation of PKCδ, and E-cadherin relocalization to cell-cell contacts was critical for LPA-induced enhanced airway epithelial barrier function [232] (Fig. 2). The role of LPA in regulation of airway barrier function in airway inflammatory disease, such as acute lung injury, needs further investigation.

7. Conclusions and future directions

In this review, we summarized the mechanisms of LPA in regulating gene expression and barrier function in human airway epithelial cells. The study of the biological role of LPA in airway inflammatory diseases is an area yet to be well defined. The recent studies reveal that LPA regulates biological responses in airway epithelial, smooth muscle, fibroblast, and lymphocyte cells. Our focus is on the role of LPA in regulation of airway epithelial cytokine production, lipid mediator release and airway epithelial barrier function since airway epithelium is the first site that responds to the increase of LPA levels in BALF of asthmatic and IPF patents. LPA induces gene expression through LPA-Rs mediated intracellular signaling, such as changes of $[Ca^{2+}]_i$, activation of PKC isoforms, PLD, and transcriptional factors (NF-κB, AP-1, and C/EBPβ). Cross-talk between LPA-Rs and RTKs (EGF-R, PDGF-Rβ, and c-Met) regulate LPA-induced gene expression, cell proliferation, and airway epithelial barrier function. LPA upregulates Th1 type cytokine (such as IL-8) and PGE2 release and attenuates the effects of Th2 type cytokine (IL-13) by releasing of an IL-13 decoy receptor, IL-13Rα2, in airway epithelial cells. This indicates that LPA in the airway may play a protective role in inflammatory diseases. Furthermore, LPA increases airway epithelial integrity through PKC isoforms-mediated E-cadherin and c-Met protein complex assembly at cell-cell contacts, suggesting that LPA protects airway by regulation of cytokine releases and airway epithelial integrity (Fig. 2). While a majority of LPA receptors are coupled to heterotrimeric G protein, recently G-protein-independent signaling through GPCRs has been demonstrated [233]. Whether LPA induces part of intracellular signaling in the absence of G-proteins in airway epithelial cells will be further investigated. Furthermore, intravenous injection with LPA attenuated bacterial endotoxin-induced plasma TNFα production and myeloperioxidase activity in lung, suggesting an anti-inflammatory role of LPA in a murine model of acute lung injury [234]. To further understand the role of LPA from both plasma and BALF in airway inflammatory diseases, the lysoPLD transgenic mice and lung epithelial cells specific lysoPLD transgenic mice will serve as useful models. As there are six receptors for LPA, development of specific antagonists for each of the receptor subtype will allow therapeutic targeting of LPA-Rs that regulate inflammation and remodeling in airway diseases.

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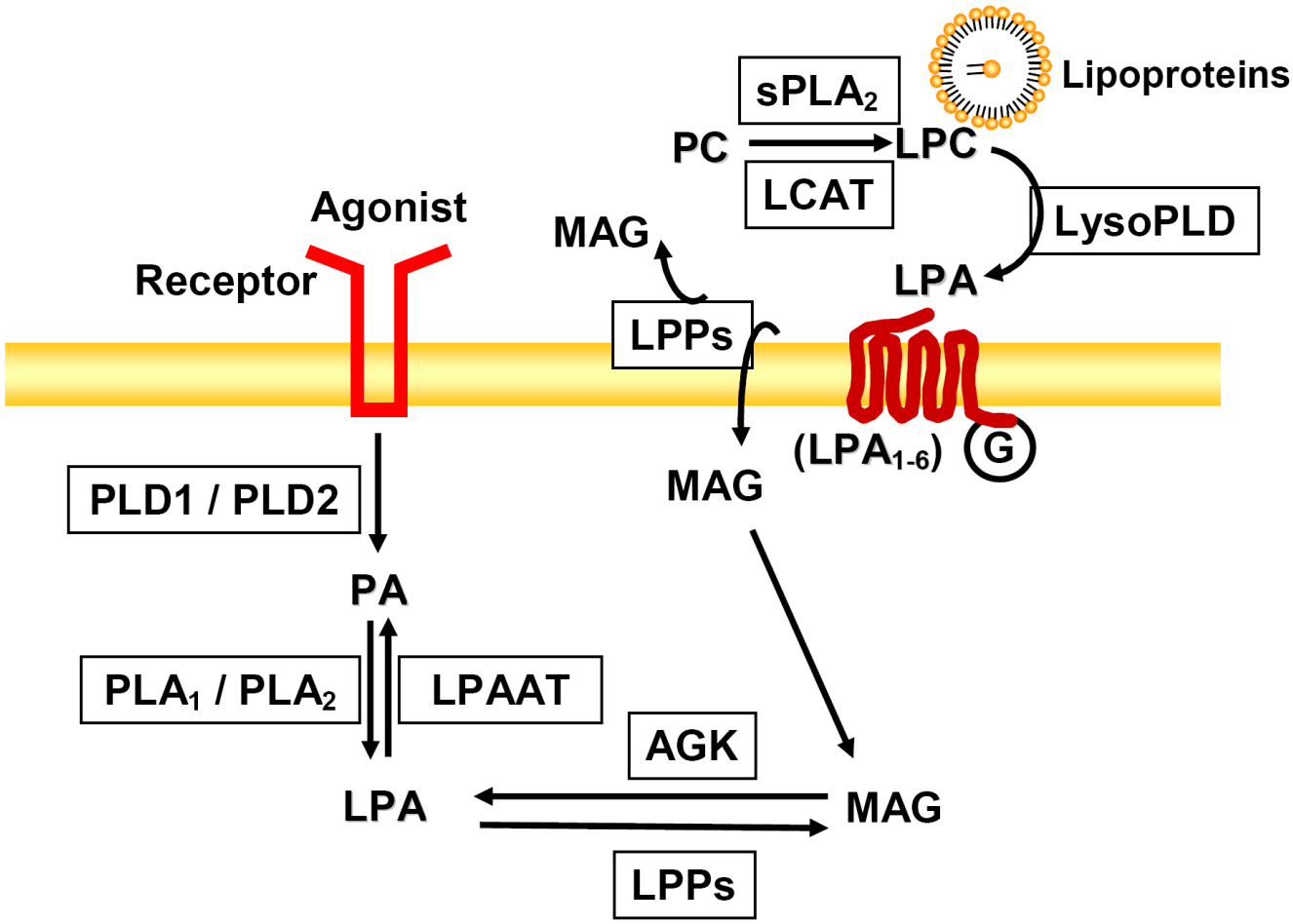

Figure 1. Biosynthesis and catabolism of LPA

Activation of PLD generates PA, which is converted to LPA by the action of PLA_1/PLA_2. MAG is converted to LPA by AGK. PC in lipoproteins serves as a substrate of sPLA2 or LCAT, which converts PC to LPC. LPC serves as a substrate of lysoPLD for LPA generation. Levels of LPA are also regulated by LPPs and LPAAT.

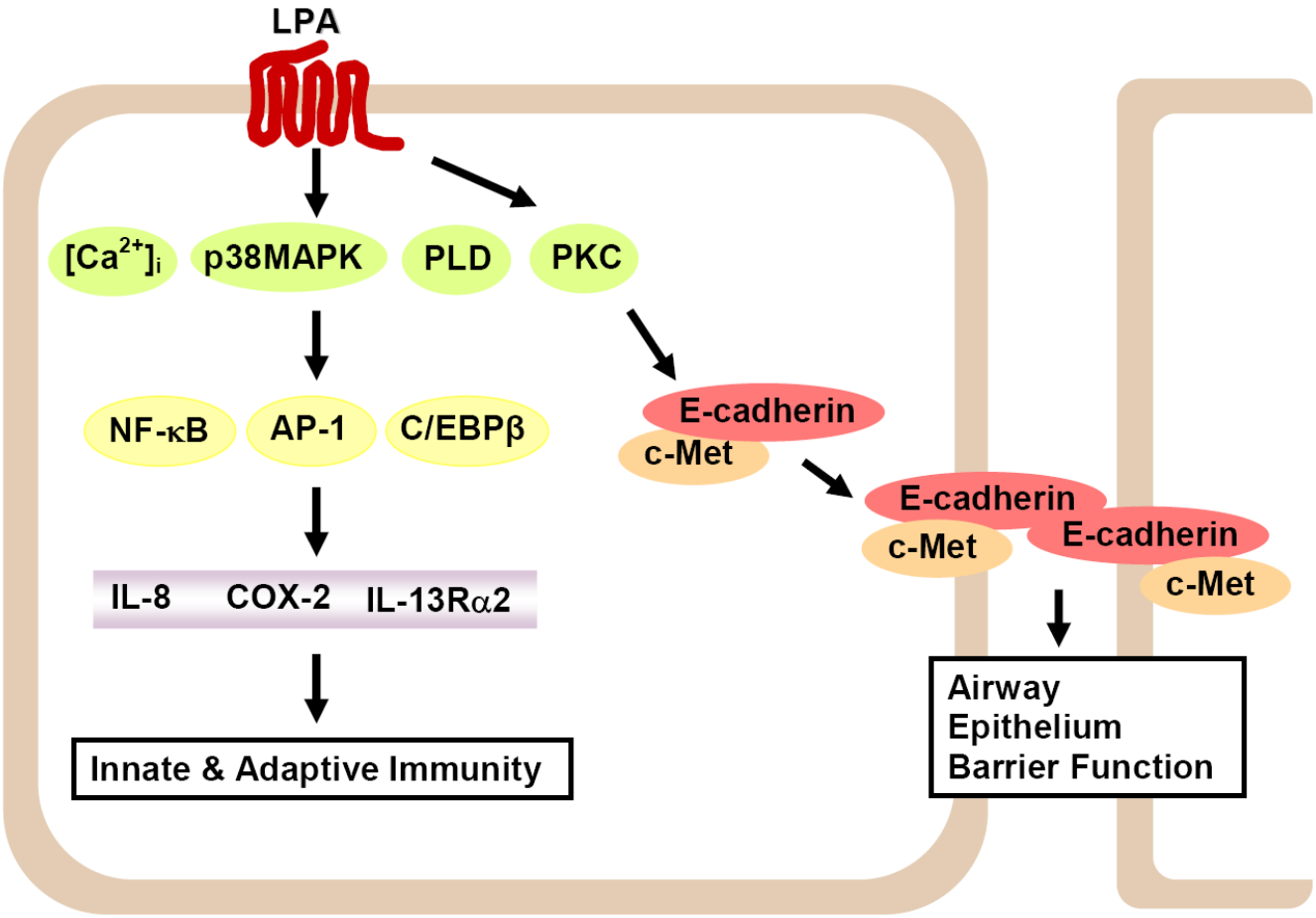

Figure 2. LPA signaling in airway epithelial cells

Ligation of LPA to LPA receptors increases intracellular calcium, activates p38 MAPK, PKCs and PLD, resulting in the activation of transcriptional factors and induction of cytokine(s) expression and release; thus regulating innate and adaptive immune responses. LPA induces E-cadherin and c-Met redistribution to cell periphery and regulates airway epithelium integrity.

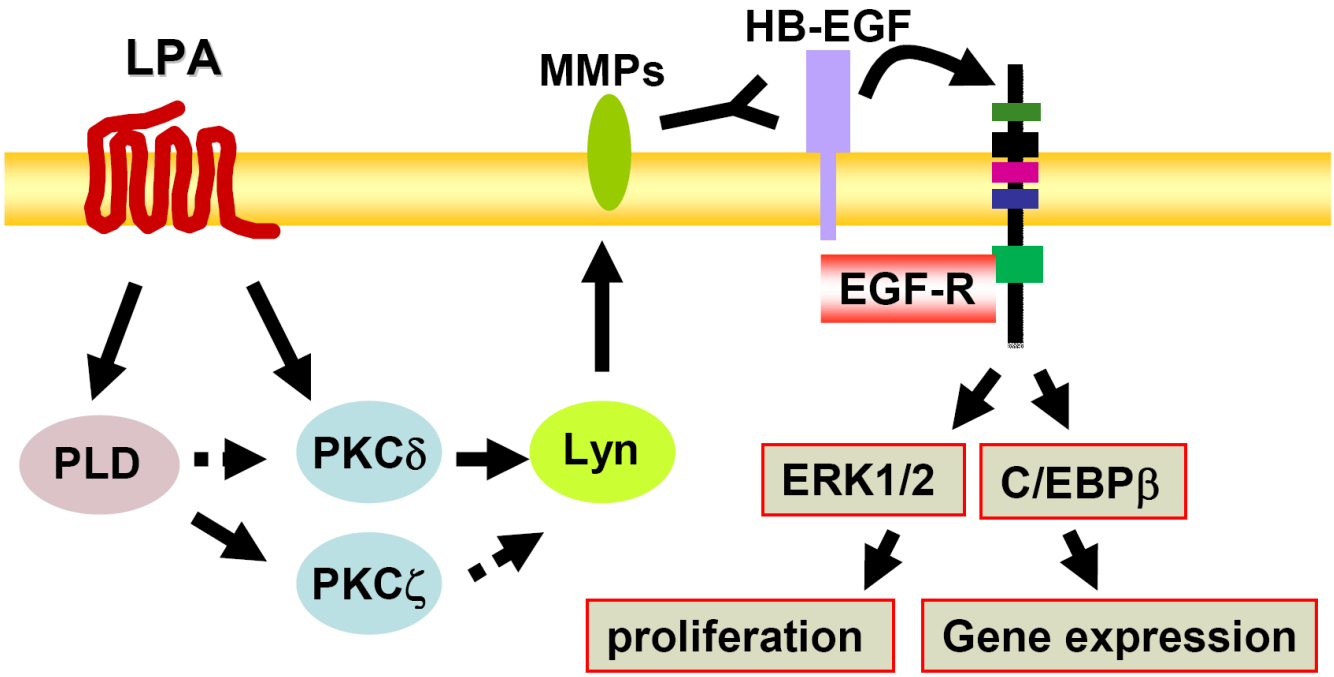

Figure 3. Signaling pathways of LPA-induced transactivation of EGF-R

Ligation of LPA to its G-protein-coupled receptors induces phosphorylation of EGF-R through PKCδ, Lyn kinase, MMP, and pro-HB-EGF pathway. PLD2 and PKCζ are involved in LPA-induced transactivation of EGF-R; however, the relationship between PLD2 and PKCδ or between PKCζ and Lyn kinase are unclear. Erk1/2 and C/EBPβ are downstream targets of EGF-R transactivation, which regulate cell proliferation and gene expression.

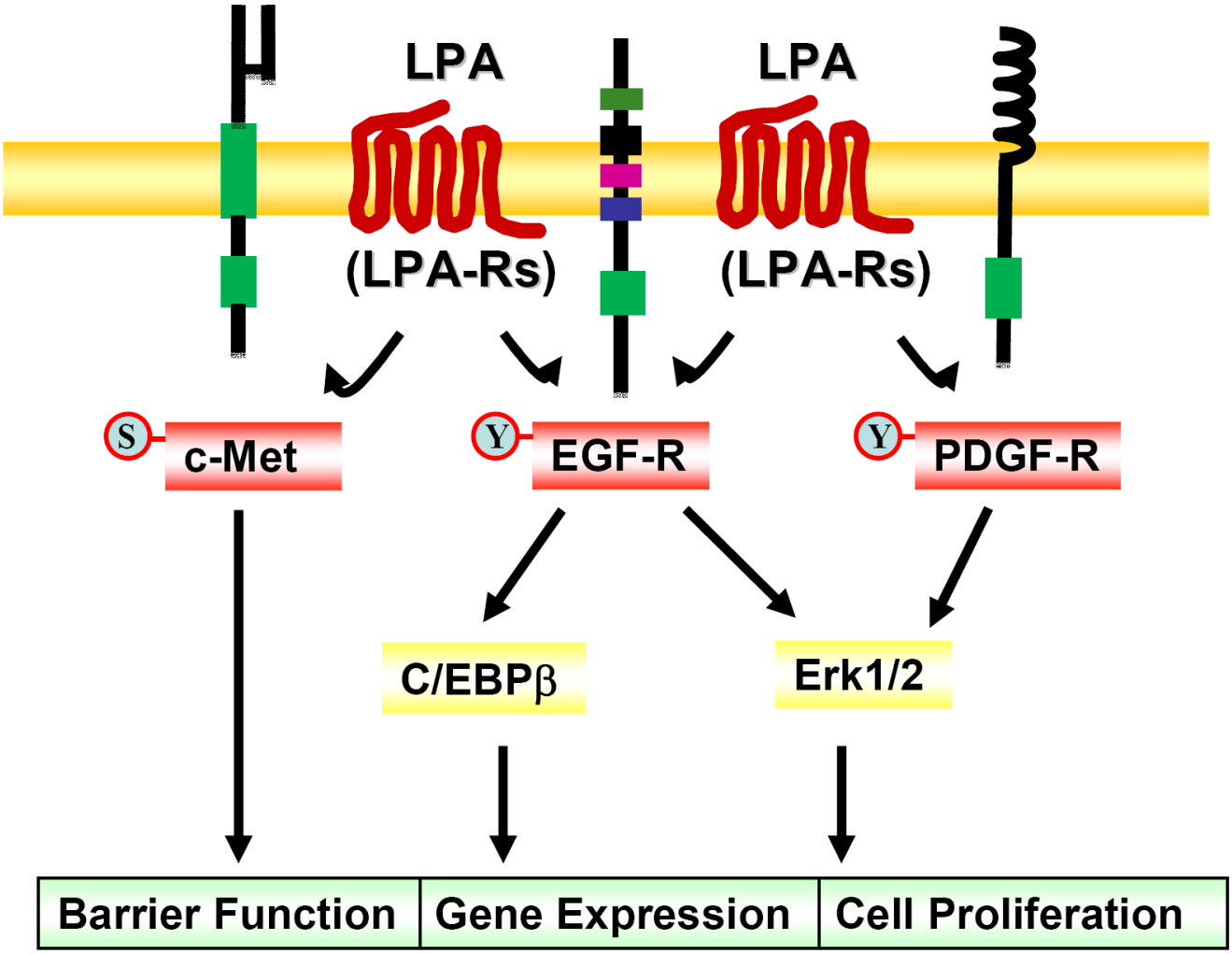

Figure 4. Cross-talk between LPA receptors and RTKs

LPA via its receptors induces tyrosine phosphorylation of EGF-R and PDGF-Rβ (termed as transactivation), and regulates cell proliferation and gene expression. Cross-talk between LPA receptors and c-Met induces serine phosphorylation of c-Met (termed as transinactivation), which enhances airway epithelial barrier function.