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Lysophosphatidic Acid Modulates c-Met Redistribution and Hepatocyte Growth Factor / c-Met Signaling in Human Bronchial Epithelial Cells Through PKC δ and E-cadherin

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Abstract

Previously we demonstrated that ligation of lysophosphatidic acid (LPA) to G protein-coupled LPA receptors induces transactivation of receptor tyrosine kinases (RTKs), such as platelet-derived growth factor receptor beta (PDGF-R β) and epidermal growth factor receptor (EGF-R), in primary cultures of human bronchial epithelial cells (HBEPs). Here we examined the role of LPA on c-Met redistribution and modulation of hepatocyte growth factor (HGF) / c-Met pathways in HBEPs. Treatment of HBEPs with LPA induced c-Met serine phosphorylation and redistribution to plasma membrane, while treatment with HGF induced c-Met internalization. Pretreatment with LPA reversed HGF-induced c-Met internalization. Overexpression of dominant negative (Dn)-PKC δ or pretreatment with Rottlerin or Pertussis toxin (PTx) attenuated LPA-induced c-Met serine phosphorylation and redistribution. Co-immunoprecipitation and immunocytochemistry showed that E-cadherin interacted with c-Met in HBEPs. LPA treatment induced E-cadherin and c-Met complex redistribution to plasma membranes. Overexpression of Dn-PKC δ attenuated LPA-induced E-cadherin redistribution and E-cadherin siRNA attenuated LPA-induced c-Met redistribution to plasma membrane. Furthermore, pretreatment of LPA attenuated HGF-induced c-Met tyrosine phosphorylation and downstream signaling, such as Akt kinase phosphorylation and cell motility. These results demonstrate that LPA regulates c-Met function through PKC δ - and E-cadherin in HBEPs, suggesting an alternate function of the cross-talk between G-protein coupled receptors (GPCRs) and RTKs in HBEPs.

Keywords

lysophosphatidic acid; G-protein coupled receptor; receptor tyrosine kinase; c-Met; E-cadherin; PKC; signal transduction

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1. Introduction

In eukaryotes extracellular messages are transduced into intracellular signals by activation of several classes of receptors. These include G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), cytokine receptor-activated kinases, and members of the steroid/thyroid hormone receptor super family [1-3]. Originally, these receptors were thought to act independently, but in recent years evidence of cross-talk between these different classes of receptors has been discovered [4-9]. Among them, the cross-talk between GPCRs and RTKs has been most frequently identified [5-9]. Activation of GPCRs leads to stimulation of RTKs, providing integration and amplification of signaling pathways that regulate cellular responses and functions [5-9]. In contrast to transactivation of RTKs by GPCRs, increasing evidence shows that GPCRs also induce inhibition of RTK activity, known as *transinactivation* [10-11].

Lysophosphatidic acid (LPA), a bioactive lysophospholipid, mediates a wide range of cellular responses via GPCRs, LPA₁ (EDG-2), LPA₂ (EDG-4), LPA₃ (EDG-7), and LPA₄ [12-15]. We have previously shown that human bronchial epithelial cells (HBEPs) express LPA₁₋₃ and that exposure to LPA stimulated expression and secretion of interleukin-8 (IL-8). The IL-8 expression is also partly regulated by protein kinase C δ (PKC δ), Nuclear factor- κ B (NF- κ B), and c-Jun-N-terminal Kinase (JNK)/AP-1 transcriptional factors [16-18]. Furthermore, LPA induced IL-13 receptor alpha2 (IL-13R α 2) expression and secretion and attenuated IL-13-induced signaling in HBEPs [19]. In addition, ligation of LPA to LPA-Rs transactivated RTKs, such as Platelet-Derived Growth Factor Receptor beta (PDGF-R β) [20] and Epidermal Growth Factor Receptor (EGF-R) [21]. EGF-R transactivation by LPA contributed to LPA-induced IL-8 secretion [21], while PDGF-R β transactivation was involved in LPA-induced phosphorylation of extracellular signal-regulated kinase (Erk) [20]. LPA also induced PDGF-R β transactivation through phospholipase D 2 (PLD2) [19] and EGF-R transactivation through PKC δ -mediated activation of lyn kinase, matrix metalloproteinases, and heparin-binding EGF (HB-EGF) shedding in HBEPs [21].

c-Met, a proto-oncogene product, is the receptor for hepatocyte growth factor (HGF), and is predominantly expressed in various types of epithelium [22,23]. Increased expression and activation of c-Met is associated with tumor invasion and metastasis [23,24]. c-Met has been shown to be expressed in several non-small cell lung cancer cell (NSCLC) lines, and activation by HGF stimulates cell growth, scattering, and invasion of these cells [24,25]. Ma et al. have identified particular mutations in the juxtamembrane and semaphorin domains, and have shown regulation of the cytoskeleton through manipulation of the juxtamembrane mutations of c-Met, especially as related to the focal adhesion protein paxillin [26]. Similar to other RTKs, ligation of HGF to c-Met induced c-Met tyrosine phosphorylation, dimerization, recruitment of several signaling molecules, and internalization [22-25,27]. Selective small molecule c-Met inhibitors (SU11274 and PHA665752) inhibited HGF-induced phosphorylation of c-Met, S6K, and Akt in NSCLC cells [28,29].

It is well established that many transmembrane receptors, such as EGF-R and c-Met, become internalized upon ligand stimulation [30-33]. It has been reported that upon HGF stimulation, c-Met is rapidly internalized via clathrin-coated vesicles and traffics through an early endosomal compartment, which is independent of the proteasome-mediated degradative pathway [34]. Kermorgant et al. showed that PKC α and PKC ϵ control c-Met traffic from plasma membrane to perinuclear compartment and this internalization plays a positive role in c-Met signal output in HeLa cells [35]. In contrast, Gandino et al. showed that PKC activation inhibited tyrosine phosphorylation of c-Met in the gastric carcinoma cell line GTL-16 [36]. Recently, Hashigasako et al. demonstrated that HGF-dependent tyrosine phosphorylation of c-Met was largely suppressed with a reciprocal relationship to Ser-985 phosphorylation by

PKC δ in A549 cell line [37]. Similar to c-Met, insulin receptors were phosphorylated at serine residues and tyrosine kinase activity was decreased by PKC [38,39]. These results suggest that PKC isoforms may play bi-directional roles in regulating c-Met signaling.

Recently, LPA-and sphingosine-1-phosphate (S1P)-induced c-Met tyrosine phosphorylation has been reported in human colon cancer cell lines and human gastric cancer cells [40,41]. Nath et al. reported that shedding of c-Met was regulated by LPA-mediated EGF-R transactivation and TIMP-3 sensitive metalloproteinase in A549 cells [37]. The role of LPA in regulating the HGF / c-Met pathways is still not clear. Here we report that LPA, through its GPCRs, stimulates serine phosphorylation of c-Met in HBEPs. LPA treatment induced c-Met redistribution to plasma membrane involving PKC δ and E-cadherin while also attenuated HGF-mediated c-Met internalization. Furthermore, LPA downregulated HGF / c-Met-mediated signaling and cell motility. These results suggest cross-talk between LPA-Rs and c-Met and a possible role of this transactivation in signal integration and epithelial cell function.

2. Materials and Methods

2.1. Materials

1-oleoyl (18:1) LPA and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Antibody to phosphoserine was from Zymed Laboratories Inc. (South San Francisco, CA). Antibodies to c-Met and E-cadherin were from Upstate (Lake Placid, NY). Antibody to PKC δ was purchased from BD Transduction Laboratories (San Jose, CA). Antibodies to total Akt and phosphor (Ser 473)-Akt were procured from Cell Signaling Technology (Beverly, MA). E-cadherin siRNAs and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Scrambled siRNA was from Dharmacon Inc. (Lafayette, CO). Pertussis toxin (PTx), Rottlerin, and bisindolylmaleimide (BIM) were from Calbiochem (San Diego, CA). HGF and anti phosphor-tyrosine-c-Met (Y1230/1234/1235) antibody were purchased from BIOSOURCE International (Camarillo, CA). Alexa fluor-488 goat anti-mouse and Alexa fluor-568 goat anti-rabbit were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from Molecular Probes (Eugene, OR). ECL kit for detection of proteins by Western blotting was obtained from Amersham Pharmacia (Piscataway, NJ). Transfection reagents were from Qiagen (Valencia, CA). Bronchial epithelial cell basal medium (BEBM) and supplement kit were purchased from Cambrex Bio Science Inc. (Walkersville, MD). All other reagents were of analytical grade.

2.2. Cell culture

HBEPs were isolated from normal human lung obtained from lung transplant donors following previously described procedures [16]. The isolated passage 0 (P₀) HBEPs were cultured in serum free basal essential growth medium (BEGM) and supplemented with growth factors. Cells were incubated at 37 °C in 5 % CO₂ and 95 % air to approximately 80 % confluence and subsequently propagated in 100 mm dishes or 6-well collagen-coated plates or cover slips. All experiments were carried out between passages 1 and 3.

2.3. Preparation of cell lysates, immunoprecipitation, and Western blotting

Cells were rinsed twice with ice-cold PBS and lysed in 500 μ l of buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β -glycerophosphate, 1 mM MgCl₂, 1 % Triton X-100, 1 mM sodium orthovanadate, 10 μ g/ml protease inhibitors, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin. Cell lysates were incubated at 4 °C for 10 min, sonicated on ice for 20 seconds and centrifuged at 5,000 g for 5 min at 4 °C in a microfuge. Protein concentrations were determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL) using BSA as standard. Equal amounts of protein (300-500 μ g) were incubated

with anti-c-Met or anti-E-cadherin antibody (2 µg/ml) overnight at 4°C. This was followed by the 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 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 again centrifuged at 5,000 g for 5 min. The supernatants were subjected to SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membranes, blocked with 5 % (w/v) BSA in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl and 0.1 % Tween-20) for 1 h and incubated with primary antibodies in 5 % (w/v) BSA in TBST for 1-2 h at room temperature. The membranes were washed at least three times with TBST at 15 min intervals and then incubated with either mouse or rabbit horseradish peroxidase-conjugated secondary antibody (1: 3,000) for 1 h at room temperature. The membranes were developed with enhanced chemiluminescence detection system according to manufacturer's instructions.

2.4. Infection with adenoviral constructs

Infection of HBEPcs (~50 % confluence) with purified adenoviral empty vector or adenoviral vector containing catalytically inactive mutant of human PKC δ (Dn-PKC δ) were carried out in 100 mm-dishes as described previously [16]. Cells were infected with different MOI in 1 ml of BEGM for 48 h.

2.5. Transfection of small interfering RNA (siRNA) for E-cadherin

HBEPcs (P1) cultured onto 6-well plates or cover slips (40-50 % confluence) were transiently transfected with human E-cadherin and scrambled siRNA using Transmessenger Transfection Reagent. Briefly, siRNAs (50 nM) were condensed with Enhancer R and formulated with Transmessenger Reagent, according to the manufacturer's instruction. The transfection complex was diluted into 900 µl of BEBM medium and added directly to the cells. The medium was replaced with BEBM medium after 3 h, and cells were used for experiments at 72 h after transfection.

2.6. Immunocytochemistry

HBEPcs grown on cover slips to approximately 70-80 % confluence were challenged with LPA or HGF. Coverslips were rinsed with PBS and treated with 3.7 % formaldehyde in PBS at room temperature for 20 min. After washing with PBS, coverslips were incubated in blocking buffer (1 % BSA in TBST) for 1 h. Cells were then subjected to immunostaining with antibody to c-Met or E-cadherin (1: 200 dilution) for 1 h, washed three times with TBST, and stained with Alexa Fluor 568 or 488 (1: 200 dilution in blocking buffer) for 1 h. After washing at least three times with TBST, the coverslips were mounted using commercial mounting medium for fluorescent microscopy (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and were examined by immunofluorescent microscope with Hamamatsu digital camera using a 60x oil immersion objective and MetaVue software.

2.7. Cell motility assay

HBEPcs were subcultured on 35 mm dishes in low density. Cells grew to separate colonies and were treated with or without LPA (1 µM) for 15 min, then challenged with HGF (25 ng/ml) for 3 h. Images were taken using microscopy with Nikon camera and analyzed by NIH ImageJ software.

2.8. Statistical analyses

All results were subjected to statistical analysis using one-way ANOVA and, whenever appropriate, analyzed by Student-Newman-Keuls test. Data are expressed as means $\pm$ S.D. of

triplicate samples from at least three independent experiments and level of significance was taken to $P < 0.01$.

3. Results

3.1. LPA induces serine phosphorylation of c-Met via G_i and PKC δ in HBEPcs

We have previously shown transactivation of RTKs, such as PDGF-R β and EGF-R, by LPA-Rs in HBEPcs [19,20]. Here we examined the cross-talk between LPA-Rs and c-Met in HBEPcs. HBEPcs were challenged with LPA (1 μ M) for 2, 15, and 30 min or HGF (50 ng/ml) for 15 min and c-Met was immunoprecipitated with an antibody specific to c-Met. As shown in Fig. 1A, LPA treatment induced serine phosphorylation of c-Met at 2 min, and for up to 30 min, while HGF had no effect on serine phosphorylation of c-Met. To examine the tyrosine phosphorylation of c-Met, the cell lysates were analyzed by Western blotting with phospho-tyrosine (Y1230/1245/1246) antibody. HGF (50 ng/ml, 15 min) induced tyrosine phosphorylation of c-Met while LPA (1 μ M) induced marginal tyrosine phosphorylation of c-Met at 15 min (Fig. 1B). To determine whether LPA-induced serine phosphorylation of c-Met is through G-protein-coupled LPA receptors, HBEPcs were challenged with LPA (1 μ M) for 15 min with or without pretreatment with PTx (100 ng/ml, 4h), then c-Met was immunoprecipitated with a c-Met specific antibody and analyzed with phosphoserine antibody. As shown in Fig. 1C, PTx attenuated LPA-induced serine phosphorylation of c-Met, suggesting LPA-induced c-Met serine phosphorylation is dependent on LPA-R-coupled G_i protein.

Our previous studies showed that PKC δ plays a critical role in LPA-induced IL-8 expression [16] and EGF-R transactivation [20]. Here we examined the role of PKC δ in cross-talk between LPA-Rs and c-Met in HBEPcs. HBEPcs were infected with empty control adenoviral vector (20 MOI) or adenoviral vector containing Dn-PKC δ (1, 10, and 20 MOI) for 48 h. The overexpression of Dn-PKC δ was confirmed by Western blotting with antibody specific to PKC δ (Fig. 2A). The effect of overexpression of Dn-PKC δ on LPA-induced serine phosphorylation of c-Met was determined by immunoprecipitation with a c-Met specific antibody and analyzed with phosphoserine antibody. As shown in Fig. 2B, Overexpression of Dn-PKC δ (1, 10, and 20 MOI) attenuated LPA-induced serine phosphorylation of c-Met in a dose-dependent fashion. Further, PKC δ inhibitor, Rottlerin blocked LPA-induced serine phosphorylation of c-Met (Fig. 2C). These results suggest that PKC δ regulates LPA-induced serine phosphorylation of c-Met in HBEPcs.

3.2. LPA induces c-Met redistribution via PKC δ in HBEPc

HGF induces c-Met endocytosis and intracellular traffic has been demonstrated in variety of cell types [33-35]. Here we examined the c-Met redistribution by LPA treatment in HBEPcs. HBEPcs (~80 - 90% confluence) grown in cover slips were challenged with LPA (1 μ M) for 15, 30, and 60 minutes. c-Met redistribution was examined by immunofluorescence with antibody to c-Met. As shown in Fig. 3A, LPA (1 μ M) treatment induced c-Met redistribution to cell plasma membrane in a time-dependent fashion in HBEPcs.

We previously showed that LPA treatment induced phosphorylation of PKC δ and translocation of PKC δ to plasma membrane [16]. To further determine the mechanisms of LPA-induced c-Met redistribution to plasma membrane, we examined the role of PKC δ in regulating c-Met traffic in response to LPA treatment. HBEPcs were treated with 0.1 % DMSO or PKC inhibitor bisindolylmaleimide I (BIM, 1.0 μ M) for 1 h, then challenged with LPA (0.1, 1.0, and 5.0 μ M) for an additional 15 min. As shown in Fig. 3B, LPA induced c-Met redistribution to plasma membrane in a dose-dependent fashion, while BIM blocked the effect of LPA on c-Met redistribution. Overexpression of Dn-PKC δ (1, 10, 20 MOI) attenuated LPA (1 μ M, 15 min)-induced c-Met redistribution in a dose-dependent fashion (Fig. 3C). PKC α has been known

to be involved in HGF-induced c-Met internalization in HeLa cells [35], while overexpression of Dn-PKC α and Dn-PKC λ had no effect on LPA-induced c-Met redistribution (Y. Zhao and V. Natarajan, unpublished data). These results suggest that PKC δ regulates LPA-induced c-Met redistribution to plasma membrane in HBEPcs.

3.3. Role of E-cadherin in LPA-induced c-Met redistribution in HBEPcs

Co-localization of E-cadherin, a cell-cell adhesion molecule, and c-Met in cell-cell adhesion sites has been shown in MDCK cells [43]. In HBEPcs co-localization of E-cadherin and c-Met in plasma membrane and cytoplasm were detected by double immunostaining with anti-E-cadherin and anti-c-Met antibodies. LPA (1 μ M) treatment induced both E-cadherin and c-Met complex redistribution to plasma membrane in a time course (Fig. 4A). Further, E-cadherin was immunoprecipitated from HBEPcs with or without LPA (1 μ M, for 10 and 30 min) treatment. As shown in Fig. 4B, interaction of E-cadherin and c-Met were found in E-cadherin complex from either LPA-treated and untreated HBEPcs. Similar to the effect of Dn-PKC δ on LPA-induced c-Met redistribution, overexpression of Dn-PKC δ (20 MOI, 48 h) attenuated LPA-induced E-cadherin redistribution to plasma membrane in HBEPcs (Fig. 5). These results suggest that LPA induced E-cadherin and c-Met complex redistribution through PKC δ in HBEPcs.

Next we examined whether LPA-induced c-Met redistribution to plasma membranes required interaction with E-cadherin. To downregulate E-cadherin expression, HBEPcs were transfected with scrambled and E-cadherin specific siRNAs (50 nM) for 72 h. As shown in Fig. 6A, LPA induced c-Met redistribution in the cells transfected with scrambled siRNA, while E-cadherin siRNA attenuated LPA-induced c-Met redistribution. The effect of E-cadherin siRNA transfection on E-cadherin protein expression was confirmed by Western blotting with anti E-cadherin antibody (Fig. 6B).

3.4. The effect of LPA on HGF / c-Met-mediated c-Met internalization, signaling, and cell motility

Consistent with others' results [33-35], in HBEPcs HGF (25 ng/ml) treatment for 30 min induced c-Met internalization from plasma membrane to cytoplasm and perinuclear compartment in a dose-dependent fashion (Fig. 7A). To further determine the effect of LPA on c-Met traffic, HBEPcs were pretreated with LPA (1 μ M) for 15 min, then challenged with 25 ng/ml of HGF for an additional 30 min. LPA pretreatment attenuated HGF-induced c-Met internalization (Fig. 7A), suggesting that LPA-induced c-Met redistribution to plasma membrane reversed HGF-induced c-Met internalization.

To determine the role of LPA treatment in c-Met-mediated signaling, HBEPcs were pretreated with or without LPA (1 μ M) for 15 min, then were challenged with HGF (25 ng/ml) for 15, 30, 60, or 120 min. HGF induced tyrosine (1230/1245/1246) phosphorylation of c-Met at 15 min and up to 120 min. Pretreatment of HBEPcs with LPA (1 μ M) inhibited HGF-induced tyrosine phosphorylation of c-Met at all the time points (Fig. 7B). To further examine the effect of LPA on HGF-mediated c-Met tyrosine phosphorylation, HBEPcs were pretreated with LPA (1 μ M) for 15 min, then challenged with HGF (10 and 25 ng/ml) for 30 min. As shown in Fig. 7C, LPA pretreatment completely abrogated 10 ng/ml of HGF-induced tyrosine phosphorylation of c-Met, and attenuated the effect of 25 ng/ml of HGF treatment by around 50 % (Fig. 8C). In addition to the effect on HGF-induced phosphorylation of c-Met, we examined the effect of LPA pretreatment on HGF / c-Met-mediated phosphorylation of Akt. As shown in Fig. 8A, HGF (25 ng/ml) induced phosphorylation of Akt at 30 min and up to 120 min. Pretreatment with LPA (1 μ M) attenuated HGF / c-Met-mediated phosphorylation of Akt kinase at 30, 60, and 120 min. These results suggest that LPA attenuates HGF-induced c-Met activation and HGF / c-Met-mediated signaling in HBEPcs.

HGF has been known to stimulate cell motility in a variety of cell types [23,24]. Without treatment, HBEPs were in contact with each other as a colony. LPA (1 μ M) treatment for 3 h increased cell-cell interaction and cell clustering while HGF (25 ng/ml) treatment for 3 h stimulated cells to separate and move around. Pretreatment with LPA (1 μ M, 15 min) inhibited HGF-induced cell motility (Fig. 8B, C). These results suggest that LPA pretreatment of HBEPs attenuated HGF-induced phosphorylation of c-Met and Akt and cell motility.

4. Discussion

We previously identified the transactivation of PDGF-R β and EGF-R by LPA ligation to G-protein-coupled LPA-Rs and determined that the transactivation of RTKs regulates LPA-mediated cellular responses, such as phosphorylation of Erk and IL-8 gene expression and secretion in HBEPs [20,21]. The present study provides the first evidence of cross-talk between LPA-Rs and c-Met and the role of this cross-talk in regulating the HGF / c-Met pathway in HBEPs. Ligation of LPA to LPA-Rs induces serine phosphorylation of c-Met and redistribution of c-Met to plasma membrane through activation of PKC δ and interaction with E-cadherin in HBEPs. HGF / c-Met mediated phosphorylation of c-Met and Akt and cell motility is partly down-regulated by LPA.

c-Met is the prototype member of RTKs and the high-affinity receptor for HGF [22,23]. Ligation of HGF to the extracellular domain of c-Met results in receptor dimerization, phosphorylation of multiple tyrosine residues at the intracellular region, and binding and activation of signal transducers such as PI-3-Kinase, PLC, STATs, and Erk1/2 [22,25-27]. Transactivation of RTKs by GPCRs induces tyrosine phosphorylation of RTKs and hence results in further signal transduction [5-9]. We have shown that LPA induced tyrosine phosphorylation of EGF-R and PDGF-R β in HBEPs [20,21]. Here we present evidence that c-Met is present in HBEPs and that LPA induces serine phosphorylation and redistribution of c-Met to plasma membrane, while it has no effect on tyrosine phosphorylation of c-Met. c-Met has been known to be transactivated by LPA or SIP receptors in human colon cancer cell lines and human gastric cancer cells [40,41]. Our results here show the cross-talk between LPA-Rs and RTKs is not limited to tyrosine phosphorylation of RTKs.

The mechanisms of LPA-induced c-Met traffic have been determined in the present study. Ligand-dependent endocytosis of RTKs was the common mechanism of receptor inactivation and recycling [30-35,44,45]. Consistent to the study in HeLa cells [35], the binding of HGF to c-Met triggers internalization and redistribution of c-Met to the perinuclear compartment in HBEPs. In contrast to the HGF treatment, LPA induces c-Met traffic to plasma membrane and attenuates HGF-mediated “traffic in” of c-Met. Kermorgant et al. showed that PKC α regulates HGF-mediated c-Met “traffic in” [35], while the mechanisms of c-Met “traffic out” by LPA has not been studied. We have determined that PKC δ played a central role in LPA-induced EGF-R transactivation [21] as well as IL-8 gene expression and secretion in HBEPs [16]. Here we present evidence that LPA-induced c-Met serine phosphorylation and redistribution to plasma membrane is dependent on activation of PKC δ in HBEPs. These results are consistent with the study in A549 cells, in which PKC δ regulates ser-985 phosphorylation of c-Met [37]. In conclusion, PKC isoforms play a bi-directional regulatory role of c-Met intracellular traffic: PKC α regulates “traffic in”, and PKC δ regulates “traffic out”. In addition to the role of PKC δ in regulation of c-Met traffic, the present study also determines the role of interaction of E-cadherin and c-Met in LPA-induced c-Met redistribution. E-cadherin, a cell surface adhesion molecule, inhibits ligand-dependent activation of diverse receptor tyrosine kinases such as Neu, EGF-R, insulin growth factor-1 receptor (IGF-1R), and c-Met [46]. Association of c-Met with E-cadherin has been reported in human colon, breast, and prostate cancer cell lines [47-49]. In HBEPs, c-Met interacts with E-cadherin and the disassociation of c-Met from complex of c-Met and E-cadherin attenuates

LPA-mediated c-Met redistribution to plasma membrane. These results suggest that interaction with E-cadherin is necessary for LPA-induced c-Met “traffic out”. Furthermore, the present study determines that LPA-induced c-Met / E-cadherin complex redistribution to plasma membrane is through PKC δ in HBEPs. In HaCaT cells, PKC δ is involved in mediating cell-cell contacts via E-cadherin [50]. Further investigation is necessary to determine the mechanisms of regulation of E-cadherin by PKC δ in HBEPs.

HGF / c-Met signaling is involved in a variety of cellular responses, particularly cell motility, migration, and invasion, which are triggered by tyrosine phosphorylation of c-Met in response to HGF [23,24]. Recently, serine phosphorylation of c-Met by PKC has been associated with decreased tyrosine phosphorylation of c-Met and has been shown to negatively regulate HGF / c-Met biological function [36,37]. Unlike LPA-mediated tyrosine phosphorylation of PDGF-R β and EGF-R [20,21], LPA only induces serine, not tyrosine or threonine, phosphorylation of c-Met in HBEPs. The present study shows that LPA down regulates HGF / c-Met-mediated phosphorylation of c-Met and Akt and cell motility in HBEPs. These results suggest that in the same type of cells, the same GPCR ligand treatment might induce “yin and yang” responses on different RTKs. The association between transactivation of EGF-R and PDGF-R β and transinactivation of c-Met by LPA are not clear. The mechanisms of downregulation of HGF / c-Met signaling need further investigation. It is possible that serine phosphorylation of c-Met: 1) changes conformation of c-Met, resulting in a decrease of ligand binding affinity; or 2) recruits tyrosine phosphatase to decrease tyrosine phosphorylation of c-Met and adaptor proteins.

Our findings have implications for biologic processes associated with LPA-induced c-Met transinactivation. Our results demonstrate that LPA-induced c-Met serine phosphorylation and redistribution through PKC δ and E-cadherin and LPA negatively regulates HGF / c-Met signalings in HBEPs. Airway epithelium plays a critical role in innate immunity. Our observations suggest that the cross-talk between LPA-Rs and c-Met may contribute to the airway protective role of LPA in innate immunity.

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Abbreviations

LPA	lysophosphatidic acid
HGF	hepatocyte growth factor
HBEPcs	human bronchial epithelial primary cells
GPCR	G-protein-coupled receptor
RTK	receptor tyrosine kinase
PDGF-Rβ	platelet-derived growth factor receptor beta
EGF-R	epidermal growth factor receptor
siRNA	small interference RNA
MOI	multiplicity of infection
Dn	dominant negative
PKC	protein kinase C
PTx	pertussis toxin
IL	interleukin
PBS	phosphate buffer solution
BIM	

bisindolylmaleimide

MOI

multipul of infection

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Figure 1. LPA induces serine phosphorylation of c-Met

A. HBEPs (70-80 % confluence in 100 mm dishes) were treated with LPA (1 μ M) for 2, 10, or 30 min or HGF (25 ng/ml) for 10 min. Cell lysates were immunoprecipitated with anti-c-Met antibody and blotted with anti-phosphoserine and anti-c-Met antibodies. **B.** HBEPs (70-80 % confluence in 35 mm-dishes) were treated with LPA (1 μ M) for 2, 10, or 30 min or HGF (25 ng/ml) for 10 min. Cell lysates were analyzed by Western blotting with anti-phosphotyrosine (1230/1245/1246)-c-Met and anti-c-Met antibodies. **C.** HBEPs (70-80 % confluence in 100 mm dishes) were pretreated with or without PTx (100 ng/ml) for 4 h, then further challenged with or without LPA (1 μ M) for 10 min. Lysates were immunoprecipitated with anti-c-Met antibody and blotted with anti-phosphoserine and anti-c-Met antibodies. Shown are representative blots of three independent experiments.

Figure 2. Overexpression of Dn-PKC δ or rottlerin attenuates LPA-induced serine phosphorylation of c-Met

HBEPcs (50 % confluence in 100 mm dishes) were infected with adenoviral empty vector (20 MOI) or adenoviral vector containing Dn-PKC δ (1, 10, and 20 MOI) for 48 h or pretreated with DMSO (0.1 %) or Rottlerin (5 μ M) for 1 h, then challenged with LPA (1 μ M) for 15 min. Cell lysates were either processed for blotting with anti-PKC δ antibody (**A**), or immunoprecipitated with anti-c-Met antibody and blotted with anti-phosphoserine and anti-c-Met antibodies (**B and C**). Shown are representative blots of three independent experiments.

Figure 3. LPA induces c-Met intracellular traffic in HBEpCs

A. HBEpCs (70-80 % confluence in cover slips) were treated with LPA (1 μ M) for 15, 30, or 60 min, and immunostained with anti-c-Met antibody. **B.** HBEpCs (70-80 % confluence in cover slips) were pretreated with BIM (1 μ M) or 0.1 % DMSO for 1 h, then challenged with LPA (0.1, 1.0, and 5.0 μ M) for an additional 15 min. Cells were immunostained with anti-c-Met antibody. **C.** HBEpCs (50 % confluence in cover slips) were infected with adenoviral empty vector (20 MOI) or adenoviral vector containing Dn-PKC δ (1, 10, and 20 MOI) for 48 h. Cells were challenged with LPA (1 μ M) for 15 min. Cells were immunostained with anti-c-Met antibody. Shown are representative images of three independent experiments.

Figure 4. c-Met associates with E-cadherin in HBEPs

A. HBEPs (70-80 % confluence in cover slips) were treated with LPA (1 μ M) for 5, 15, or 30 min, and double immunostained with anti-c-Met (green) and anti-E-cadherin (red) antibodies. Combined images shown in lower pattern. Shown are representative images of three independent experiments. **B.** HBEPs (70-80 % confluence in 100 mm dishes) were treated with LPA (1 μ M) for 10 or 30 min. Lysates were immunoprecipitated with anti-E-cadherin antibody and blotted with anti-E-cadherin or anti-c-Met antibody. Shown are representative blots of three independent experiments.

Figure 5. Overexpression of Dn-PKC δ attenuates LPA-mediated E-cadherin redistribution in HBEpCS

HBEpCS (50 % confluence in cover slips) were infected with adenoviral empty vector (20 MOI) or adenoviral vector containing Dn-PKC δ (20 MOI) for 48 h. Cells were challenged with or without LPA (1 μ M) for 15 min. Cells were immunostained with anti-E-cadherin antibody. Shown are representative images of three independent experiments.

Figure 6. E-cadherin siRNA attenuates LPA-induced c-Met redistribution in HBEpCs

A. HBEpCs (50 % confluence in cover slips) were transfected with scrambled and E-cadherin siRNA (100 nM) for 72 h, then challenged with or without LPA (1 μ M) for 15 min. Cells were immunostained with anti-c-Met antibody. Shown are representative images of three independent experiments. **B.** HBEpCs (50 % confluence in 6-well plates) were transfected with scrambled and E-cadherin siRNA (100 nM) for 72 h. Lysates were blotted with anti-E-cadherin or anti-c-Met antibody. Shown are representative blots of three independent experiments.

Figure 7. LPA attenuates HGF-induced tyrosine phosphorylation of c-Met

A. HBEPcs (70-80 % confluence in cover slips) were treated with or without LPA (1 μ M) for 15 min, then challenged with or without HGF (25 ng/ml) for an additional 30 min. Cells were immunostained with anti-c-Met antibody. Shown are representative images of three independent experiments. **B.** HBEPcs (70-80 % confluence in 6-well plates) were pretreated with or without LPA (1 μ M) for 15 min, then challenged with HGF (25 ng/ml) for 15, 30, 60, or 120 min. Cell lysates were blotted with anti-phospho-c-Met or anti-c-Met antibody. Shown are representative blots of three independent experiments. **C.** HBEPcs (70-80 % confluence in 6-well plates) were pretreated with or without LPA (1 μ M) for 15 min, then challenged with HGF (10 and 25 ng/ml) for 30 min. Cell lysates were blotted with anti-phospho-c-Met and anti-c-Met antibodies. Shown are representative blots of three independent experiments.

Figure 8. LPA attenuates HGF / c-Met-mediated signaling and cell motility

A. HBEPcs (70-80 % confluence in 6-well plates) were pretreated with or without LPA (1 μ M) for 15 min, then challenged with HGF (25 ng/ml) for an additional 30, 60, or 120 min. Cell lysates were blotted with anti-phospho-Akt and anti-Akt antibodies. Shown are representative blots of three independent experiments. **B.** HBEPcs colonies (grown on 100 mm dishes) were pretreated with or without LPA (1 μ M) for 15 min, then challenged with or without HGF (25 ng/ml) for 3 h. Shown are representative images of five independent experiments. **C.** Cells area was measured with NIH imageJ software.

Figure 9. Mechanisms of regulation of HGF / c-Met signaling by LPA in HBEPs
 Ligation of LPA to G-coupled LPA receptors induces c-Met serine phosphorylation and redistribution through activation of PKC δ and interaction with E-cadherin, resulting in attenuation of HGF / c-Met-mediated phosphorylation of c-Met and Akt and cell motility.