

15-Lipoxygenase 1 interacts with phosphatidylethanolamine-binding protein to regulate MAPK signaling in human airway epithelial cells

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Epithelial 15-lipoxygenase 1 (15LO1) and activated ERK are increased in asthma despite modest elevations in IL-13. MAPK kinase (MEK)/ERK activation is regulated by interactions of Raf-1 with phosphatidylethanolamine-binding protein 1 (PEBP1). Epithelial 15LO1 generates intracellular 15-hydroxyeicosatetraenoic acid (15HETE) conjugated to phosphatidylethanolamine (PE) (15HETE-PE). We hypothesized that (i) 15LO1 and its product 15HETE-PE serve as signaling molecules interacting with PEBP1 to activate Raf-1/MEK/ERK and that (ii) this 15LO1–15HETE-PE-regulated ERK activation amplifies IL-4R α downstream pathways. Our results demonstrate that high epithelial 15LO1 levels correlate with ERK phosphorylation *ex vivo*. *In vitro*, IL-13 induces 15LO1, which preferentially binds to PEBP1, causing PEBP1 to dissociate from Raf-1 and activate ERK. Exogenous 15HETE-PE similarly induces dissociation of PEBP1 from Raf-1 independently of IL-13/15LO1. siRNA knockdown of 15LO1 decreases the dissociation of Raf-1 from PEBP1, and the resulting lower ERK activation leads to lower downstream IL-4R α -related gene expression. Identical protein-protein interactions are observed in endobronchial biopsies and fresh epithelial cells from asthmatics *ex vivo*. Colocalization of Raf-1 to PEBP1 is low in asthmatic tissue and cells compared with normals, whereas there is striking colocalization of 15LO1 with PEBP1 in asthma. Low 15LO1 levels in normals limit its colocalization with PEBP1. The results confirm a previously unknown signaling role for 15LO1 and its PE-conjugated eicosanoid product in human airway epithelial cells. This pathway enhances critical inflammatory pathways integral to asthma pathogenesis.

eicosanoids | phospholipid | MUC5AC

Asthma is a significant health problem resulting in nearly 1 million emergency department visits and over half a million hospitalizations annually in the United States (1). Animal studies of allergic inflammation show a strong T helper type-2 (Th2) bias in association with IL-4 and/or IL-13. Although supporting human data are more limited (2), antagonizing the IL-4R α receptor reduces responses to inhaled allergen in mild asthmatics (3). In severe asthma, there is evidence for a sustained “Th2 signature” with eosinophilic inflammation (4, 5), mucus production (6–9), and up-regulation of the Th2-associated eicosanoid enzyme, 15-lipoxygenase 1 (15LO1) (5, 10, 11).

15LO1 is a key enzyme involved in fatty acid metabolism that oxidizes unsaturated fatty acids, including arachidonic acid (AA), at the 15 position to generate active hydroperoxy and epoxy metabolites (12). Recent studies confirm a critical regulatory role for 15LO1 and its product 15-hydroxyeicosatetraenoic (15HETE) in IL-13-induced mucin (MUC)5AC expression of human airway epithelial cells and further show that 15HETE is not released extracellularly but rather maintained intracellularly in a form conjugated with phosphatidylethanolamine (PE) (11). Thus, the IL-13 induction of 15LO1–15HETE-PE, their intracellular location and interactions with phospholipids as well as

15HETE-PE's contribution to MUC5AC expression suggest that the 15LO1 pathway may play a role in intracellular signaling in response to IL4R α activation.

Extracellular signal-regulated kinase (ERK), a ubiquitous mitogen-activated protein kinase (MAPK), is also increased and activated in epithelial cells from severe compared with milder asthmatics and normal controls (13). ERK activation *in vitro* is generally transient, such that the mechanism for the sustained activation of ERK observed in severe asthma is not clear. However, ERK phosphorylation occurs when its upstream activator Raf-1 dissociates from its natural inhibitor, phosphatidylethanolamine binding protein 1 (PEBP1) [also known as Raf kinase inhibitory protein (RKIP)] and is free to activate the MAPK kinase (MEK)/ERK pathway (14–16). This dissociation occurs either as a result of increased phosphorylation at S153 or displacement by phospholipids (17). Levels of this protein have been suggested to play critical roles in tumor suppression (18, 19) and Alzheimer's disease (20, 21) by sustaining phosphorylation of ERK, but have not been studied in lung disease.

Because IL4R α engagement activates ERK (22, 23), as well as induces expression of 15LO1–15HETE-PE (11), and because both are increased in human asthmatic epithelial cells, it was hypothesized that 15LO1 and its lipid product 15HETE-PE would competitively interact with PEBP1, contribute to the prolonged activation of ERK, and critically regulate downstream signaling pathways integral to asthma. Accordingly, interactions of 15LO1–15HETE-PE with the PEBP1–Raf-1-binding complex and subsequent MAPK/ERK activation were investigated *ex vivo* in fresh human airway epithelial cells from asthmatic and control subjects and *in vitro* in primary human epithelial cells stimulated with IL-13 in air liquid interface (ALI) cultures. The results identify a critical role for the 15LO1 pathway in the activation and regulation of MAPK signaling.

Results

Subject Demographics. Fresh airway epithelial cells were obtained from a total of 65 subjects (Table S1). Due to the limited number of cells available, not all cells were used for both *ex vivo* and *in vitro* experiments. Studies of freshly harvested epithelial cells were performed on cells from 56 subjects (20 severe, 20 mild–moderate asthmatics, 16 normal controls). Cell culture studies were performed on cells from 40 subjects (13 severe asthmatics, 10 mild–moderate asthmatics, and 17 normal controls). Although there were substantial differences of 15LO1 expression

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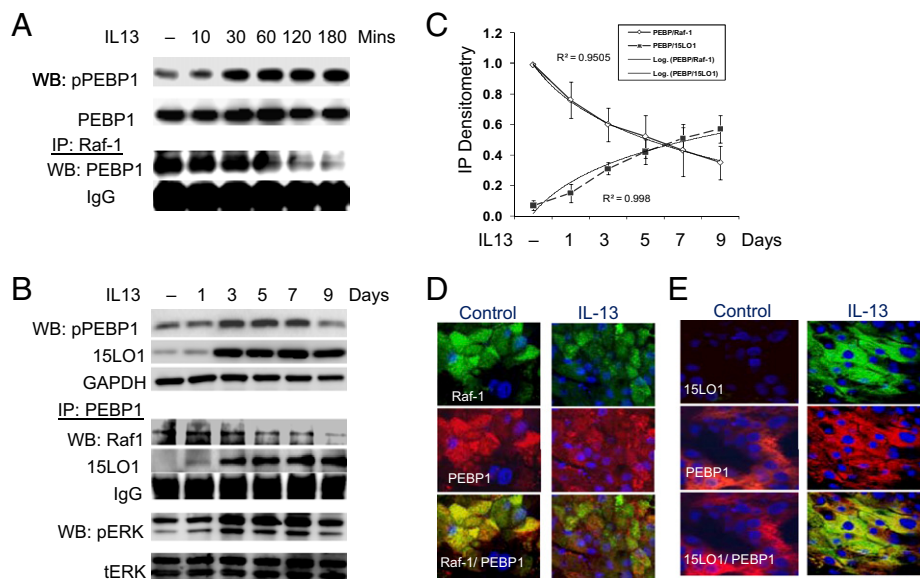


Fig. 3. IL-13-induced-15LO1 amplifies MEK/ERK pathways through interaction with PEBP1-Raf-1. ALI-cultured primary human bronchial epithelial cells were stimulated with IL-13 for different time periods. Total protein was harvested for IP/Western blot. (A) Acute stimulation of IL-13-induced PEBP1 phosphorylation and PEBP1-Raf-1 dissociation in a time-dependent manner. Cells were cultured for 7 d under ALI and then stimulated with acute IL-13 for different times. (B) Prolonged IL-13 stimulation-induced 15LO1 interacts with PEBP1-Raf-1 to activate ERK. Cells under ALI culture were stimulated with IL-13 for different times before total protein was harvested for IP/Western blot. (C) Regression analysis of the IP densitometry data demonstrates the increased PEBP1 binding to 15LO1 and the decreased Raf-1 binding over time. Immunofluorescence/confocal analysis showing (D) decreased binding of PEBP1 and Raf-1 and (E) increased 15LO1 expression and colocalization of 15LO1 to PEBP1 induced by IL-13. Data are representative of at least three independent experiments.

results strongly suggest that prolonged IL-13-induced 15LO1 competitively inhibits the binding of Raf-1 to PEBP1, freeing Raf-1 to enhance ERK activation.

15LO1 Product 15HETE-PE also Competitively Inhibits Raf-1 Binding to PEBP1 and Activates ERK. Epithelial 15HETE is not released in a “free” form, but rather esterified to phospholipids (i.e., PE) as 15HETE-PE (11). To determine whether the interaction of 15LO1 with PEBP1 pathway was specific to 15LO1 protein itself or due to interactions with its product 15HETE-PE, exogenous 15HETE-PE was added in the absence of IL-13 (where there is low level basal 15LO1). Similar to the findings with IL-13 stimulation, addition of 15HETE-PE competitively decreases Raf-1 binding to PEBP1 in association with an increase in pERK, compared with the vehicle control dimyristoyl-phosphatidylethanolamine (DMPE). This effect is seen in the absence of significant phosphorylation of PEBP1 (Fig. 4). No significant 15LO1 is induced by 15HETE-PE stimulation. Thus, these results suggest that, in the absence or near absence of 15LO1, its enzyme product 15HETE-PE also interacts with PEBP1 to release Raf-1 and activate ERK. As specificity control experiments, neither 18:0/18:0-PE, lyso-PE, nor semipurified 5HETE-PE (60% 5HETE-PE, 40% 9HETE-PE) significantly induced ERK phosphorylation and PEBP1-Raf-1 displacement (Fig. S2).

Inhibition of 15LO1 Expression Prevents Dissociation of PEBP1 from Raf-1 Suppressing IL-13-Induced ERK Activation and MUC5AC, Induced Nitric Oxide Synthase, and Eotaxin 3 Expression. Cells in early ALI culture were transfected with ALOX15 siRNA and stimulated with IL-13 for 48 h. As shown in Fig. 5A, ALOX15 siRNA transfection significantly knocked down IL-13-induced 15LO1 protein compared with scramble siRNA control. No effects on 15LO2 expression were observed (Fig. S3). This decrease in 15LO1 limits its availability for binding to PEBP1. With diminished levels of 15LO1 following ALOX15 transfection, the dissociation of PEBP1 from Raf-1 lessens and ERK activation decreases compared with scramble siRNA. To confirm the biologic relevance of the interaction of 15LO1 with PEBP1, MUC5AC, induced nitric oxide synthase (iNOS), and eotaxin 3 expression was measured by quantitative real-time PCR. As reported previously (11, 25) and as shown in Fig. 5B, IL-13-induced MUC5AC, iNOS, and eotaxin 3 are significantly inhibited by siRNA knockdown of 15LO1 ($P < 0.01$). These results confirm that interaction of 15LO1-15HETE-PE with PEBP1 regu-

lates IL-13-induced MAPK/ERK activation and could further modify downstream gene expression of relevance to asthma.

15LO1 and PEBP1 Interactions Occur Primarily at the Cell Membrane.

Both PEBP1 and Raf-1 are membranous lipid-binding proteins and membrane-associated signaling proteins. 15LO1 metabolizes membrane lipids and interacts with membrane phospholipids such as PE (24). Thus, it was hypothesized that PEBP1, Raf-1, and 15LO1 interactions occur in the cell membrane. To test this, subcellular fractions from cells chronically stimulated with IL-13 (10 d) were isolated for IP and Western blot detection of PEBP1, Raf-1, 15LO1, and ERK. As shown in Fig. 6A, IL-13 induces 15LO1 protein predominantly in the cytosol but also in membrane fractions. IP of the total protein demonstrates decreased binding of PEBP1 to Raf-1 and increased 15LO1 binding to PEBP1 following IL-13 (also shown in Fig. 3). IP of the protein fractions confirms that the majority of the decrease in binding of PEBP1 to Raf-1 occurs in the membrane fraction. Concomitant with this decrease, the increase in PEBP1 binding to 15LO1 is seen predominantly in the membrane fraction, and the majority of the 15LO1 protein remains in the cytosolic fraction. High-amplification confocal microscopy confirms dissociation of PEBP1 from Raf-1, and PEBP1 colocalizes with 15LO1 in the membrane following IL-13 stimulation (Fig. 6B). Thus, the majority of the interactions between PEBP1 and either Raf-1 or

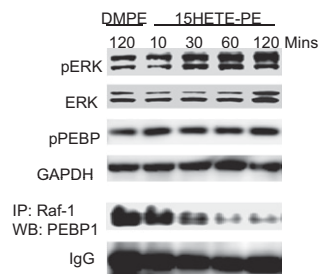


Fig. 4. 15HETE-PE competitively inhibits Raf-1 binding to PEBP1, leading to phosphorylation of ERK. Primary human bronchial epithelial cells under ALI culture were stimulated with 1 μ M 15HETE-PE. IP/Western blot was performed as previously described. DMPE was applied as the vehicle control and incubated with the cells for 2 h. Data are representative of at least three independent experiments.

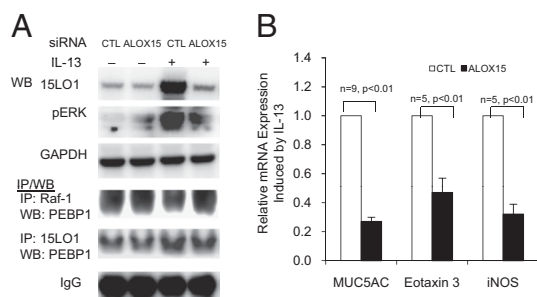


Fig. 5. ALOX15 siRNA (and associated lower 15 LO1) limits the dissociation of PEBP1 from Raf-1, thereby suppressing ERK activation and MUC5AC, iNOS, and eotaxin 3 gene expressions induced by IL-13. ALI-cultured cells transfected with ALOX15 siRNA were stimulated with IL-13 for 48 h. The total proteins were harvested for IP/Western blot analysis and mRNA was harvested for real-time PCR. Data are representative of at least three independent experiments. (A) Inhibition of 15LO1 by siRNA transfection suppressed ERK activation induced by IL-13. Co-IP and Western blot demonstrate that knockdown of 15LO1 diminished its binding to PEBP1 while lessening the dissociation of PEBP1 from Raf-1 induced by IL-13. (B) IL-13-induced expressions of MUC5AC, iNOS, and eotaxin 3 were significantly inhibited by ALOX15A siRNA knockdown of 15LO1 in vitro.

15LO1 occur at the cell membrane, although the specific membrane location remains to be determined. Notably, 15LO1 also translocates into the nucleus with IL13 stimulation, but the significance of this is not yet known.

15LO1 Competes with Raf-1 to Bind with PEBP1 ex Vivo in Asthmatic Cells and Tissue. To determine whether the interactions between 15LO1–15HETE–PE and PEBP1–Raf-1 observed in vitro reflect the pathobiologic process occurring in the airways of asthmatics, freshly brushed bronchial epithelial cells from both asthmatic and normal subjects were evaluated per in vitro protocol by IP/Western blot (Fig. 7). As expected, 15LO1 and ERK phos-

phorylation are higher in asthmatic compared with normal subjects. IP with fresh epithelial cell protein demonstrates increased binding of PEBP1 to 15LO1 and less binding to Raf-1 in asthmatic compared with normal control subjects. Confirming the IP results, confocal images of endobronchial tissue demonstrate decreased colocalization of Raf-1 to PEBP1 in a severe asthmatic compared with a normal control subject without difference in total Raf-1 and PEBP1 protein expression (Fig. 8A). In contrast, the high level of epithelial 15LO1 protein in the severe asthmatic is strikingly colocalized with PEBP1, whereas low-level 15LO1 expression limits the colocalization of 15LO1 to PEBP1 observed in the normal control (Fig. 8B).

Discussion

The results reported here identify a critical signaling role for epithelial 15LO1 and its product 15HETE–PE in asthma and potentially in other diseases, including cancers (26–28) and atherosclerosis (29, 30) where 15LO1 is also expressed. 15LO1 expression/activation associates with increased activation of ERK both in vitro and in vivo in human airway. In response to acute IL-13 stimulation, the initial activation of IL4R α leads to PEBP1 phosphorylation, which releases Raf-1 from PEBP1 and activates ERK in the absence of 15LO1. However, after prolonged stimulation, IL-13 induced 15LO1–15HETE–PE competitively dissociates Raf-1 from PEBP1 to sustain and even augment ERK activation independently of PEBP1 phosphorylation, enhancing downstream gene expression. These in vitro findings are mirrored ex vivo by identical changes in fresh asthmatic epithelial cells and endobronchial biopsy specimens. This study identifies a unique interaction of 15LO1–15HETE–PE with PEBP1 that regulates IL-4R α signaling and gene expression through the MAPK/ERK pathway. Finally, these interactions observed ex vivo strongly support immediate biologic relevance of these interactions to human diseases such as asthma.

15LO1 is a key eicosanoid enzyme involved in fatty acid metabolism. Both 15LO1 and one of its products, 15HETE, have been observed to be increased in asthma and related to eosinophilic inflammation (5, 10). In addition, 15LO1 is one of the genes most strongly induced by IL-13 (31–33). An important role for the 15LO1 pathway in asthmatic inflammatory processes is further suggested by the recent confirmation of its critical role in MUC5AC expression from both human (11) and 12/15LO1^(-/-) murine studies (34, 35). Exogenous 15HETE also enhances IL-13-induced MUC5AC expression in human airway epithelial cells (11) and stimulates mucus in rat trachea (36). However, the mechanism by which 15LO1 regulates MUC5AC expression remains unknown. In contrast to the proinflammatory role of 15LO1 in murine asthma models, data from a 15LO1-over-expressing rabbit model suggested an anti-inflammatory role of 15LO1 during atherosclerosis (37, 38). Whether this difference is due to the disease or the animal model remains to be clarified.

Unlike other eicosanoids, 15LO1 does not appear to function through receptor-mediated mechanisms, and, indeed, no receptor has been identified. A lipid interaction study in dendritic cells showed that 15LO1 binds to phospholipids [e.g., phospho-

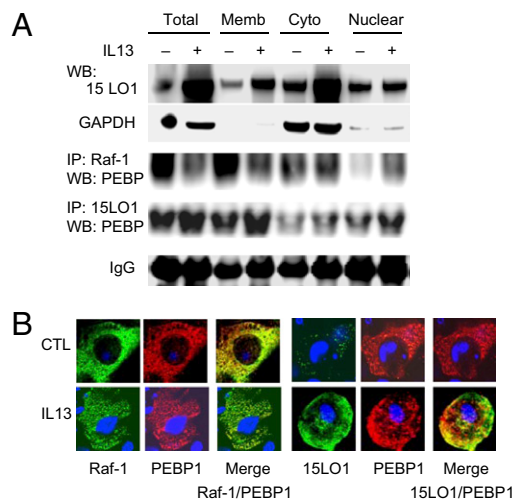


Fig. 6. Interactions of 15LO1 and PEBP1 occur primarily at the cell membrane. ALI-cultured primary bronchial epithelial cells were stimulated with IL-13 for 10 d, and subcellular fractions were harvested for IP/Western blot. (A) IL-13 induced 15LO1 expression primarily in the cytosol with lower amounts in the membrane fractions. IP/Western blot showed that the majority of the decrease of Raf-1 binding and the subsequent increase of 15LO1 binding to PEBP1 occurred in the membrane fraction. (B) High-amplification confocal microscopy confirmed the decrease in Raf-1 colocalization with PEBP1 and the increase in 15LO1 colocalization with PEBP1 in the cell membranes following IL-13 stimulation. Data are representative of at least three independent experiments.

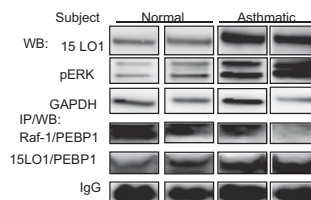


Fig. 7. 15LO1 competes with Raf-1 to bind with PEBP1 ex vivo in asthmatic cells and tissue. Fresh bronchial epithelial cells from both normal and asthmatic subjects were processed for IP/Western blot as described in *Materials and Methods*. Data are representative of at least three independent experiments.

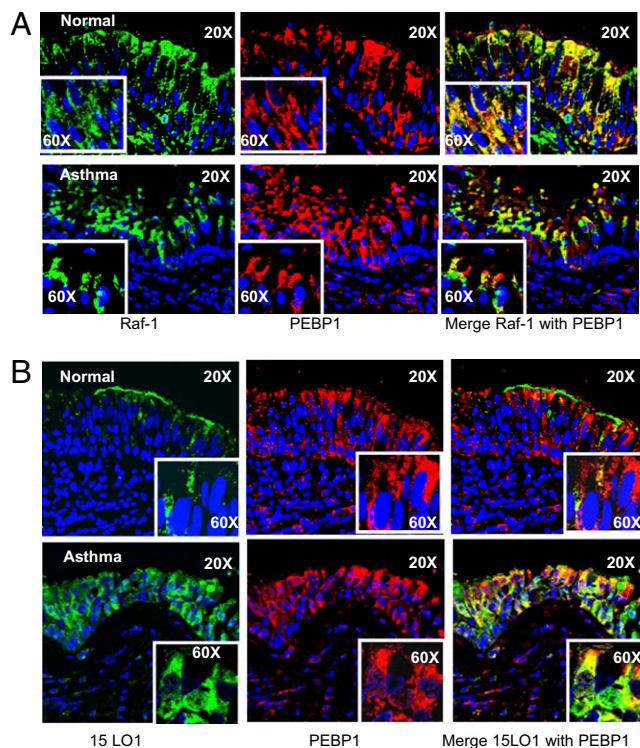


Fig. 8. Confocal images of endobronchial tissue demonstrate that 15LO1 competes with Raf-1 to bind with PEBP1 *ex vivo* in asthmatic tissue. Endobronchial biopsies fixed in acetone, embedded in glycol-methacrylate for IF staining, observed under the Olympus Fluoview 1000 confocal microscope, and analyzed using Metmorph software. (A) Markedly less colocalization of Raf-1 to PEBP1 in severe asthmatic compared with normal control tissue. (B) High expression levels and colocalization of 15LO1 with PEBP1 in severe asthmatic compared with normal control tissue.

tidylinositol (PIs) and certain PEs], which then stimulate generation of 15HETEs and 13-hydroxyoctadecadienoic acids (13HODEs) (24). Earlier studies showed acute addition of exogenous 15HETE was incorporated into intracellular PIs in neutrophils (39) and epithelial cells (40). Because only about 10% of the 15HETE-PI was detectable 48 h later, its role over longer periods of time is not clear. In contrast, studies in monocytes showed that endogenously generated 15HETEs conjugated with intracellular PE (41), and, in primary human airway epithelial cells, IL-13-induced endogenous 15HETE-PE is intracellular (11). These differences in the conjugation of 15HETE support the presence of varying biologic processes dependent on cell type and exogenous addition or endogenous generation of 15HETE and its conjugates. Because phospholipids have significant roles in signal transduction and gene expression, interactions between 15LO1-15HETE and phospholipids (PE here) have the potential to modify various signaling pathways.

PEBPs were originally identified as a class of proteins that specifically bind the phospholipid PE (42). Two of these PEBP proteins (PEBP1 and -4) have been identified in humans. PEBP1, also known as RKIP, was later identified to inhibit Raf-1-mediated MAPK signaling through interaction with Raf-1 (14). Under homeostatic conditions, PEBP1 binds to Raf-1 and inhibits Raf-1-mediated MAPK signaling by preventing phosphorylation of Raf-1 (14). Phosphorylation of PEBP1 at Ser153 by protein kinase C induces a conformational change in the ligand-binding pocket and leads to dissociation of PEBP1-Raf-1 binding and activation of MEK/ERK pathways (43). The pocket appears capable of binding to chemically distinct ligands, including phospholipids and Raf-1, which further compete with the PEBP1 binding to Raf-1 and regulate MAPK (17). Dysregulation

of PEBPs is suggested to contribute to human disease pathogenesis, with intense interest in its role in Alzheimer's disease, diabetes, and cancer (review in ref. 15). However, in none of these diseases has an interaction between PEBP1 and 15LO1 pathway been reported, nor has PEBP1 been investigated in lung disease.

In the current study, IL-13 induces a time- and 15LO1 concentration-dependent decrease in binding of PEBP1 to Raf-1. Although 15LO1 was originally identified as a fatty acid metabolic enzyme, it has also been reported to bind to other molecules including phospholipids (24) and proteins (44). In the setting of asthma/Th2 stimulation where 15LO1 protein and its product 15HETE/HETE-PE are highly expressed, its interactions with PEBP1-Raf-1 could dramatically impact ERK activation. The results shown here confirm a decrease in Raf-1 binding to PEBP1, which parallels an increase in PEBP1 binding to 15LO1. This relationship is also reversed by 15LO1 knockdown, confirming that IL-13-induced 15LO1 competitively binds to PEBP1, releases Raf-1, and activates ERK. Because 15LO1 has also been reported to bind to phospholipids (24), whether 15LO1 binds to PEBP1 through the common ligand PE, or shares the same binding motif in the PEBP1 pocket with Raf-1, requires further investigation. The 15LO1 product 15HETE-PE also enhances the dissociation of Raf-1 from PEBP1 even in the absence or near absence of 15LO1. Because exogenous addition of 15HETE-PE does not appear to induce PEBP1 phosphorylation and 15LO1 expression, the dissociation of Raf-1 from PEBP1 induced by 15HETE-PE is independent of IL-13, 15LO1, and PEBP1 phosphorylation. This result is consistent with a previous study that showed that phospholipid binding in the PEBP1-binding pocket inhibits both Raf-1 binding to PEBP1 and its phosphorylation (17). Thus, 15HETE-PE could represent a naturally occurring PE-containing phospholipid capable of displacing Raf-1 from PEBP1. Because the binding pocket of PEBP1 could bind to chemically distinct ligands, including phospholipids and lipoproteins (17), this binding pocket is the most likely region where PEBP1 could interact with both 15LO1 protein and its phospholipid product 15HETE-PE. As 15LO1 can contribute to the generation of other metabolites including 13HODE and lipoxins, it remains to be determined whether they also conjugate with PE and interact with PEBP1 in the same manner as 15HETE-PE. However, we believe this to be unlikely in epithelial cells because 15HETE-PE is the only conjugated eicosanoid detected in our epithelial samples. Because lyso-PE failed to induce ERK phosphorylation and PEBP1-Raf-1 displacement *in vitro*, it is unlikely that exogenous 15HETE-PE acts by formation of this lipid in human airway epithelial cells. Whether 15HETE-PE directly binds to PEBP1 as 15LO1 does, whether PE modification with 15HETE-PE also dissociates PEBP1-Raf-1 binding, or whether 15HETE-PE shares the same binding motif with 15LO1 all require further investigation. Because 15LO1 is expressed by macrophages, eosinophils (5), and mast cells (45), whether the interaction of the 15LO1 pathway with PEBP1 is specific to epithelial cells will also need to be addressed.

PEBP1, Raf-1, and ERK itself are all membrane-associated proteins. 15LO1 metabolizes unsaturated fatty acids such as AA, initiated by phospholipase-induced hydrolysis of AA at the cell membrane (46-48). The results in the present study confirm that most of the PEBP1 binding to 15LO1 occurs in the membrane fraction despite the majority of 15LO1 remaining in the cytosolic fraction. Similar compartmental shifts are well described for other lipoxygenase enzymes (49, 50). These findings support the concept that the primary interactions between 15LO1 and PEBP1 occur at the cell membrane, although the specific membrane location remains unknown. As the cell membrane is the primary location for any number of signaling events involving phospholipids, whether the interaction of 15LO1 with PEBP1 alters other membrane-associated signaling pathways remains to be determined.

In summary, the results from the present study using primary human airway epithelial cells indicate that high levels of 15LO1 interact with PEBP1 to displace Raf-1 and sustain MAPK/ERK activation. This process is consistently observed in cell culture

in vitro and, more importantly, is present in human asthmatic epithelial cells in vivo. Thus, we have identified a proinflammatory molecular signaling pathway of immediate relevance to human disease, which could contribute to mucus hypersecretion and eosinophilic inflammation in diseases such as asthma.

Materials and Methods

Reagents and Antibodies. The 15LO1 antibody was a gift from Doug Conrad, University of California, San Diego (51). All other antibodies and reagents used are described in *SI Materials and Methods*.

Subjects, Bronchoscopy Protocol, and ex Vivo Study. Normal subjects had no history of any respiratory illness and normal pulmonary function testing. All asthmatic subjects met American Thoracic Society criteria for asthma and ranged from mild to severe (52). Bronchoscopy with endobronchial epithelial

brushing was performed as described previously (9, 53). Ex vivo studies were performed as described in *SI Materials and Methods*.

Primary Bronchial Epithelial Cell Culture and siRNA Transfection. All were performed as described in *SI Materials and Methods*.

Co-IP, Western Blotting, Immunofluorescence, and Confocal Microscopy. All were performed as described in *SI Materials and Methods*.

Data and Statistical Analysis. For details, see *SI Materials and Methods*.

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