Interleukin-13–induced MUC5AC Is Regulated by 15-Lipoxygenase 1 Pathway in Human Bronchial Epithelial Cells

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Rationale: 15-Lipoxygenase-1 (15LO1) and MUC5AC are highly expressed in asthmatic epithelial cells. IL-13 is known to induce 15LO1 and MUC5AC in human airway epithelial cells *in vitro*. Whether 15LO1 and/or its product 15-HETE modulate MUC5AC expression is unknown.

Objectives: To determine the expression of 15LO1 in freshly harvested epithelial cells from subjects with asthma and normal control subjects and to determine whether IL-13–induced 15LO1 expression and activation regulate MUC5AC expression in human bronchial epithelial cells *in vitro*.

Methods: Human airway epithelial cells from subjects with asthma and normal subjects were evaluated *ex vivo* for 15LO1 and MUC5AC expression. The impact of 15LO1 on MUC5AC expression *in vitro* was analyzed by inhibiting 15LO1 through pharmacologic (PD146176) and siRNA approaches in human bronchial epithelial cells cultured under air–liquid interface. We analyzed 15 hydroxyeicosatetraenoic acid (15-HETE) by liquid chromatography/UV/mass spectrometry. MUC5AC and 15LO1 were analyzed by real-time RT-PCR, immunofluorescence, and Western blot.

Measurements and Main Results: Epithelial 15LO1 expression increased with asthma severity ($P < 0.0001$). 15LO1 significantly correlated with MUC5AC *ex vivo* and *in vitro*. IL-13 increased 15LO1 expression and stimulated formation of two molecular species of 15-HETE esterified to phosphatidylethanolamine (15-HETE-PE). Inhibition of 15LO1 suppressed 15-HETE-PE and decreased MUC5AC expression in the presence of IL-13 stimulation. The addition of exogenous 15-HETE partially restored MUC5AC expression.

Conclusions: Epithelial 15LO1 expression increases with increasing asthma severity. IL-13 induction of 15-HETE-PE enhances MUC5AC expression in human airway epithelial cells. High levels of 15LO1 activity could contribute to the increases of MUC5AC observed in asthma.

Keywords: 15-lipoxygenase-1; MUC5AC; asthma pathogenesis; inflammation; lipid mediator

Asthma is a significant public health problem, but its pathogenesis remains poorly understood (1). Animal models and some human studies suggest a strong Th2 bias, with increases in IL-4 and/or IL-13 contributing to the process through differentiation of ciliated epithelial cells into a goblet cell/mucus-producing epithelium (2, 3). The resulting remodeling of the epithelium and associated mucus hypersecretion contribute to the overall morbidity and

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

15-Lipoxygenase-1 (15LO1) and its metabolite, 15-HETE, are highly expressed in asthma and induced by IL-13 in human airway epithelial cells *in vitro*. Mucus hypersecretion is a key feature of asthma, and MUC5AC is recognized as one of the major components secreted by human airway epithelial cells.

What This Study Adds to the Field

15LO1 expression is increased in asthmatic epithelial cells and correlates with MUC5AC expression. Induction of 15LO1 and its product 15-HETE-PE by IL-13 up-regulate MUC5AC in human airway epithelial cells. High levels of 15LO1 activity could contribute to the increased MUC5AC observed in asthma.

mortality of asthma (4–6). Of the identified mucin genes (*MUCs*), the secreted mucins *MUC5AC* and *MUC5B* appear to be the most highly expressed in the human airway epithelium, with several studies reporting an increase in MUC5AC expression in asthmatic epithelial cells (5, 7–9). IL-13 has been shown to increase epithelial cell mucus in animal models and human airway epithelium *in vitro* (10–15).

15-Lipoxygenase-1 (15LO1), one of the key enzymes in the arachidonic acid pathway, metabolizes arachidonic acid to form stable 15-hydroxyeicosatetraenoic acid (15-HETE). 15LO1 is expressed by human asthmatic airway epithelial cells, macrophages, and eosinophils. Asthmatic macrophages demonstrate greater expression and activation of 15LO1 than normal macrophages, and 15-HETE levels increase with increasing asthma severity (16, 17). 15LO1 expression is up-regulated in human airway epithelial cells and macrophages *in vitro* in response to IL-4 and IL-13, converting arachidonic acid to 15-HETE (18–22), with some of the 15-HETE produced by human monocytes esterified to phosphatidylethanolamine (PE) to form 15-HETE-PE (23). Whether human airway epithelial cells generate free or esterified 15-HETE is not known.

Despite the strong association of 15LO1/15-HETE with asthma, the function of the 15LO1 pathway is poorly understood. Two recent studies showed that inflammatory processes and airway mucus decreased after allergen challenge in 12/15LO (–/–) mice, the murine equivalent of human 15LO1 (24, 25). Based on these results and on the previous observations that 15-HETE and MUC5AC are increased in human asthmatic airways, we hypothesized that 15LO1 and its products would regulate MUC5AC expression in humans. To accomplish this, we evaluated the relationship of 15LO1 and MUC5AC expression *ex vivo* in human airway epithelial cells and determined whether 15LO1/

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15-HETE-PE could modify IL-13–induced MUC5AC expression *in vitro*.

Some of the results of these studies have been previously reported in the form of abstracts (26, 27).

METHODS

Additional details on reagents and methods are provided in the online supplement.

Reagents, Antibodies, and Primes

Calcein A23187 was purchased from Sigma (St. Louis, MO), and 15(S)-HETE was purchased from Cayman (Ann Arbor, MI). ALOX15 small interfering RNA (siRNA) was purchased from Ambion (Austin, TX), and *Mirus siQUEST* transfection reagent was from Mirus (Madison, WI). MUC5AC antibody was purchased from Thermo Fisher Scientific (Fremont, CA), and the 15LO1 antibody was a gift from Dr. Doug Conrad, University of California, San Diego (28). Real-time PCR primers and probes were purchased from Applied Biosystems (Foster City, CA).

Bronchoscopy Protocol and *Ex Vivo* Study

Bronchoscopy with endobronchial epithelial brushing was performed as described previously (9, 29). Normal subjects had no history of any respiratory illness and normal pulmonary function testing. All subjects with asthma met American Thoracic Society criteria for asthma and ranged from mild to severe (30). No subject smoked in the last year or had a >5 pack-year history of smoking. Airway brushings were processed for epithelial cell RNA and protein to evaluate 15LO1 and MUC5AC expression. Endobronchial biopsies from the lower lobe subcarinae were fixed in acetone for immunofluorescence staining using goat polyclonal antibody against 15LO1 and mouse polyclonal against MUC5AC. The results of staining were analyzed using a confocal microscope (*see online supplement for details*).

Primary Bronchial Epithelial Cell Culture under Air–Liquid Interface

Primary human bronchial epithelial cells were cultured under air–liquid interface (ALI) as previously described (9, 29, 31). From Day 0 of ALI, cells were stimulated with IL-13 (10 ng/ml) every 48 hours for up to 10 days. For the chemical inhibitor studies, the 15LO1 inhibitor (PD146176, 1 μ M; Pfizer, New York, NY) was added into the medium for 10 days cells with or without IL-13 stimulation (32, 33). For ionophore studies, A23187 (10 μ M) was added into culture 30 minutes before harvest. For exogenous 15(S)-HETE stimulation, 1 μ M unconjugated 15(S)-HETE was added to the medium at various time points before harvest, with ethanol (1%) added into the medium as the vehicle control.

siRNA Transfection of Primary Human Epithelium Cells under ALI

siRNA transfection was performed on 70% confluent cells in 12-well transwell plates during the submerged culture stage using Mirus si-QUEST transfection reagent. After transfection for 24 hours, the cells were switched to ALI culture and stimulated with IL-13 for 48 hours. To determine whether 15-HETE could rescue the inhibition, 1 μ M unconjugated exogenous 15(S)-HETE was added into the medium 24 hours before harvest. Scrambled siRNA was transfected at the same time as negative control.

Liquid Chromatography/UV/Mass Spectrometry Analysis

Cells were kept in Krebs' buffer containing diethylenetriaminepentaacetic acid (100 μ M) and butylated hydroxytoluene (100 μ M). Free and esterified HETEs were analyzed by LC/MS/MS as described in the online supplement (23, 34). Fold changes in 15-HETE-PE were determined by comparison with basal levels in each subject and are expressed as fold-change after stimulation.

Quantitative Real-Time PCR

Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems) using primers and probes from

TABLE 1. DEMOGRAPHICS OF SUBJECTS FOR STUDY

	Normal Control Subjects	Mild-Moderate Asthma	Severe Asthma	P Value
Gender, M/F	10/15	9/8	13/13	NS
Age, years	33 (28–38)	37 (31–43)	42 (37–42)	0.06
FEV ₁ %, mean $\pm$ SE	103 $\pm$ 3	88 $\pm$ 4	58 $\pm$ 4	<0.0001
ICS	0	6/17	26/26	NA
Oral GC	0	0	16/26	NA

Definition of abbreviations: GC = glucocorticoid; ICS = inhaled corticosteroid; M/F = male/female; NA = not applicable; NS = not significant.

Applied Biosystems, with GAPDH serving as the internal control. An identical threshold cycle (Ct) was applied for each gene of interest. Relative mRNA expression levels were calculated using the delta Ct method.

Western Blotting

Cell lysates were run on 12% SDS-PAGE gels under reducing conditions as previously described (29). Western blot was performed using primary antibodies against 15LO1 and GAPDH. The membrane was developed and densitometry performed using Multi Gauge Software (Fuji Digital Co., Stamford, CT).

Data and Statistical Analysis

Statistical analysis was performed using JMP SAS software (Cary, NC). Data that were normally distributed were represented as means $\pm$ SEM. Log transformation was done on variables that were heavily right skewed, producing variables that were normally distributed (15LO1, MUC5AC protein, and mRNA *ex vivo* and *in vitro*). Graphic representation of log-transformed data was done by reconvert the log values (means and standard errors) back to the original scale. Pairwise correlation analyses were performed between 15LO1 and MUC5AC expression *ex vivo* and *in vitro*. Differences between conditions and subject groups were analyzed by ANOVA on normal and log-transformed data. When there were significant differences between the conditions, specific intercondition differences were assessed by Tukey–Kramer testing. A *P* value of 0.05 was considered statistically significant.

RESULTS

Subjects Demographics

Fresh epithelial cells were obtained from a total of 68 subjects (Table 1). Due to the limited number of cells available, not all cells were used for both *ex vivo* and *in vitro* experiments. *Ex vivo* fresh epithelial cell studies were performed on protein from 33 subjects (11 patients with severe asthma [SA], 12 patients with mild-moderate asthma [MA], and 10 normal control subjects [NC]) and mRNA from 41 subjects (16 patients with SA, 9 patients with MA, and 16 NC). Cell culture studies were performed on cells from 34 subjects (12 patients with SA, 6 patients with MA, and 16 NC).

15LO1 Expression in Bronchial Epithelial Cells Increases with Asthma Severity *Ex Vivo*: Relation to MUC5AC Expression

Ex vivo epithelial cell 15LO1 protein expression was greater in subjects with asthma compared with NC ($P < 0.01$) and increased with asthma severity (Figures 1A and 1B; overall $P < 0.01$). Due to its size and complexity, MUC5AC protein was not evaluable by Western blot.

15LO1 mRNA was not increased in asthma or severe asthma ($P = 0.75$). Similarly, despite the known increase of MUC5AC protein in asthma, MUC5AC mRNA expression was not increased in fresh epithelial cells ($P = 0.98$). However, MUC5AC mRNA positively correlated with 15LO1 protein ($R = 0.49$; $n = 17$; $P < 0.05$) and mRNA ($R = 0.31$; $n = 41$; $P < 0.05$).

Immunofluorescence staining was performed on serial biopsy sections from a control subject and a subject with asthma to

Figure 1. 15LO1 expression in fresh bronchial epithelial cells increases with asthma severity *ex vivo*. (A) Representative Western blots of 15LO1 protein expression in fresh bronchial epithelial cells from normal subjects ($n = 2$) and subjects with mild ($n = 3$) and severe ($n = 3$) asthma. (B) Quantitative analysis of 15LO1 protein expression in fresh bronchial epithelial cells using densitometry, with β -actin as the loading control. All values are means $\pm$ SE.

determine whether 15LO1 and MUC5AC protein were colocalized *ex vivo*. Confocal analysis confirmed the cellular colocalization of 15LO1 and MUC5AC in epithelial cells (Figure 2).

IL-13 Induces 15LO1 Expression and Production of Intracellular 15-HETE Conjugated to Phosphatidylethanolamine *In Vitro*

IL-13 (10 ng/ml for 10 d) induced 15LO1 protein (Figures 3A and 3B; $n = 15$; $P < 0.01$) and mRNA (Figure 3C; $n = 14$; $P = 0.01$) in epithelial cells *in vitro*. The response of 15LO1 to IL-13 stimulation *in vitro* did not differ between asthmatic and control cultured epithelial cells (shown as solid and dashed lines, respectively, in Figures 3B and 3C). To extend these studies, the effect of IL-13 on the 15LO2 pathway was evaluated under similar conditions. 15LO2 did not increase with IL-13 stimulation (Figure 3D).

15LO1 generates four phosphatidylethanolamine-esterified 15-HETE lipids and free 15-HETE in ionophore-activated human monocytes (23). To determine which 15LO1 products are generated by epithelial cells, the culture supernatants and lysates with or without IL-13 treatment in the presence or absence of ionophore (A23187, 10 μ M, for 30 min before harvest) were analyzed by LC/MS/MS. In contrast to monocytes, little or no free 15-HETE was generated by epithelial cells (data not shown). To search for ions containing esterified 15-HETE, lipid extracts were analyzed using negative ion precursor scanning LC/MS/MS. Several ions were observed; however, only those around 23 minutes showed characteristic HETE internal fragments on MS/MS analysis (Figure 4A). These were found to be single-charged lipids with

m/z 738.7 and 764.8 (Figure 4B), proposed to be two plasmalogen PEs, 16:0p/15-HETE-PE and 18:1p/15-HETE-PE (Figure 4C), which were further confirmed by comparison to ions from oxidation products of brain plasmalogen phospholipid by soybean 15-LOX (Figure E1). The MS/MS spectra of epithelial lipids and those obtained from soybean 15-LOX oxidation of PE demonstrate daughter ions at m/z 219 and 175, both diagnostic ions for the 15-HETE fragment (Figure 4C). These indicate that the lipids contain the 15-HETE positional isomer and no others (e.g., 5- or 12-HETEs). Plasmalogen and 15-HETE-PE forms increased in response to IL-13 (Figure 5A). The addition of ionophore only modestly increased the levels of 15-HETE-PEs induced by IL-13 and did not induce free 15-HETE. These lipids were detected in cell extracts but not in supernatants. No significant differences in 15-HETE-PE generation were observed across subject groups.

Exogenous 15(S)-HETE stimulation modestly increased 15-HETE-PE levels in the presence of IL-13 stimulation, whereas no increase was seen in the absence of IL-13 (Figure 5B). This suggests that exogenous 15(S)-HETEs can conjugate with intracellular phospholipids under conditions of IL-13 stimulation in epithelial cells or that 15-HETE in the presence of IL-13 induces further *de novo* generation of 15-HETE-PE.

IL-13 Induces MUC5AC *In Vitro*, which Correlates with 15LO1 Expression

To confirm that IL-13 increases MUC5AC expression in the ALI system, MUC5AC mRNA expression was analyzed in the presence and absence of IL-13 (10 ng/ml, 10 d). IL-13 strongly induced MUC5AC mRNA expression in cells from 27 subjects (10 SA, 6 MA, and 11 NC) (Figure 6; $P < 0.01$). No significant differences in MUC5AC expression in response to stimulation were seen across the subject groups. The MUC5AC mRNA expression induced by IL-13 correlated with 15LO1 protein ($R = 0.63$; $n = 13$; $P < 0.05$) and mRNA ($R = 0.64$; $n = 13$; $P < 0.05$). These *in vitro* correlations further suggest interactions between MUC5AC and 15LO1.

Exogenous 15-HETE Enhances IL-13-induced MUC5AC Expression *In Vitro*

Exogenous 15(S)-HETE (1 μ M) was added to the cultured epithelial cells with or without IL-13 (10 ng/ml for 10 d), and MUC5AC mRNA expression was analyzed. MUC5AC mRNA expression by IL-13 was further enhanced with the addition of exogenous 15(S)-HETE (Figure 7). 15(S)-HETE alone did not increase MUC5AC expression in the absence of IL-13.

Pharmacologic Inhibition of 15LO1 Decreases IL-13-induced MUC5AC mRNA Expression

Primary epithelial cells were stimulated with IL-13 (10 ng/ml) for 10 days in the presence of a pharmacologic inhibitor of 15LO1 (PD146176; 1 μ M; 10 d). This 15LO1 inhibitor decreased the intracellular levels of 15-HETE-PE induced by IL-13 stimulation (Figure 8A; $P < 0.05$). Inhibition of 15LO1 (and 15-HETE-PE) by PD146176 also suppressed MUC5AC mRNA expression (Figure 8B; $n = 8$; $P < 0.05$). There were no significant differences in cellular responses across subject groups.

ALOX15 siRNA Decreases IL-13-induced MUC5AC Expression, and Exogenous 15-HETE Neutralizes this Suppression

Because pharmacologic inhibitors often have off-target effects, epithelial cells were transfected with specific 15LO1 siRNA (ALOX15 siRNA) to confirm the pharmacologic inhibitor study. IL-13-induced 15LO1 mRNA and protein were significantly decreased in cells transiently transfected with ALOX15 siRNA (3 d of transfection and then 2 d in ALI) (Figures 9A and 9B).

Figure 2. Representative immunofluorescence staining of 15LO1 and MUC5AC protein in tissues demonstrating the increases and colocalization of 15LO1 with MUC5AC in a subject with severe asthma compared with normal control subject. The results of staining were viewed using confocal microscopy. 15LO1 staining is green, MUC5AC is red, and nucleus staining is blue (Draq5 stain). Colocalization of 15LO1 and MUC5AC is displayed as yellow.

Similar to the effects of the pharmacologic inhibitor, siRNA inhibition of 15LO1 expression suppressed IL-13-induced MUC5AC expression by 60 to 85% (Figure 9C; $n = 9$; $P < 0.01$). There were no significant differences in cellular responses across subject groups. The addition of exogenous 15(S)-HETE partially restored IL-13-induced MUC5AC expression (Figure 9C; $n = 5$; $P < 0.05$) to the basal levels. Due to the necessity of early addition of the siRNA to proliferating cells and the short duration of the transfection (2 d of ALI, as compared with 10 d of ALI in other experiments), the absolute 15-HETE-PE levels were much lower than those observed in the typical 10-day experiments (20- to 50-fold lower; data not shown). Despite the significant decrease in 15LO1 after ALOX15 siRNA transfection, no significant changes in the very low levels of 15-HETE-PE were observed at this point.

DISCUSSION

The results from the present study support an important role for airway epithelial cell 15LO1 and its product, 15-HETE-PE, in the induction of MUC5AC expression in human asthma. 15LO1 protein was significantly up-regulated in asthmatic human airway epithelial cells, particularly in severe asthma. 15LO1 and MUC5AC expression were significantly correlated *ex vivo* and *in vitro*. This is the first study we are aware of that demonstrates that selective addition and removal of 15LO1/15-HETE-PE from cultured primary human airway epithelial cells regulates MUC5AC expression. Our results strongly suggest that up-regulation of epithelial 15LO1 in human asthma contributes to the associated increase in MUC5AC expression. Thus, 15LO1 and its conjugated lipid

product, 15-HETE-PE, could serve as important modifiers of the inflammatory and repair processes associated with Th2 immune processes.

Lipoxygenases are a family of lipid-peroxidating enzymes that oxidize unsaturated fatty acids, such as arachidonic acid, at specific positions (5, 8, 12, or 15) to generate active hydroperoxy and epoxy metabolites (35). The 15LO enzymes (15LO1 and 15LO2) are expressed by a variety of cells, including human airway epithelial cells, macrophages, eosinophils, and mast cells (36–38). Activation of 15LO1 or 15LO2 leads to the production of the unstable intermediate 15-hydroperoxyicosatetraenoic acid, which is metabolized to the stable 15-HETE. Human lung, bronchi, and isolated epithelial cells are capable of converting arachidonic acid to 15-HETE (19, 20). However, a recent study suggested that up to 30% of the 15-HETE produced in monocytes existed, primarily intracellularly, in a form conjugated with the fatty acid PE, which is believed to be derived from a PE-esterified arachidonic acid substrate (23).

We and others have reported 15-HETE to be increased in asthmatic airways with levels and activity significantly greater in patients with severe asthma (16, 17, 19). Additionally, a recent report suggested a significant genetic association of 15LO1 with asthma (39). However, no previous study has quantified the expression of 15LO1 in asthmatic compared with normal epithelial cells *ex vivo*. The results from the present study show that 15LO1 protein is significantly higher in bronchial epithelial cells from subjects with asthma compared with normal control subjects, being highest in the subjects with the most severe asthma. In contrast to protein, no differences were observed in 15LO1 mRNA (or

Figure 3. IL-13 induces 15LO1 expression in human bronchial epithelial cells under air-liquid interface (ALI) culture. Primary human bronchial epithelial cells were cultured with or without IL-13 (10 ng/ml) stimulation for 10 days. (A) Representative Western blots of 15LO1 protein expression induced by IL-13 (10 ng/ml for 10 d) *in vitro*. (B) Densitometric analysis of 15LO1 protein expression induced by IL-13, with GAPDH as the loading control. *Solid line*: normal control subjects. *Dashed line*: patients with asthma. (C) Quantitative real-time RT-PCR analysis of 15LO1 mRNA induced by IL-13 *in vitro*, with GAPDH as the internal control. *Solid line*: normal control subjects. *Dashed line*: patients with asthma. (D) Western blot showing no change in 15LO2 expression with IL-13 stimulation *in vitro*.

MUC5AC mRNA) among the groups, likely due to posttranscriptional and translational regulatory elements. Such discrepancies between mRNA and protein expression *in vivo* are relatively common (40–43).

Despite these consistent reports of increased 15LO1 and 15-HETE in human asthma, the role for this pathway remains obscure. Two recent studies in mice suggested that the murine equivalent of 15LO1 regulated airway inflammation and mucus in response to OVA sensitization and challenge (24, 25). Similarly,

15LO1 and MUC5AC are up-regulated in response to Th2 stimulation of cultured human airway epithelial cells (13–15, 18, 21, 22). However, no studies have determined whether expression and activation of 15LO1 regulates MUC5AC in human cells.

Although it is unlikely that the 15LO1 enzyme regulates MUC5AC expression, its product likely does. 15-HETE is not believed to cause biologic responses through a receptor-driven mechanism. Rather, 15-HETE appears to regulate intracellular signal transduction through its incorporation into membrane

Figure 4. Precursor scanning identifies esterified 15-HETEs generated by activated epithelial cells. Lipid extracts from ionophore-activated cells were separated using liquid chromatography and scanned using precursor scanning as described in METHODS. (A) Ions eluting at 23 minutes. *Characteristic HETE daughter fragments on MS/MS. (B) Spectrum of ions eluting in region marked by an asterisk in A, showing prominent ions at *m/z* 738.7 and 764.8. (C) Scheme structures of 15-HETE-PE lipids generated by human airway epithelial cells.

in Krebs buffer for LC/ESI/MS/MS analysis. All values were normalized to the corresponding basal level of each subject and expressed as the means of fold change $\pm$ SE. Two specific esterified 15-HETE-PE (738/219 and 764/219 ions) were increased in response to IL-13. (A) IL-13-induced 15-HETE-PE conjugations *in vitro*, with modest additional increase in 15-HETE-PE after ionophore stimulation. (B) Exogenous 15(S)-HETE (1 μ M, unconjugated) increased the levels of 15-HETE-PE in the presence of IL-13 stimulation.

phospholipid pools, such as the PE pool observed in these studies (44, 45). Ideally, in the studies reported here, 15 HETE-PE would have been added to determine its effects on MUC5AC expression. However, large quantities of chemical-grade or commercial 15-HETE-PE are not available. Therefore, it was important to determine whether the addition of exogenous 15-HETE increased the levels of 15-HETE-PE in human epithelial cells. Our results showed that the addition of 15-HETE (only in the presence of IL-13) induced a modest (25%) but significant increase in 15-HETE-PE. This is similar to what has been reported in epithelial cells in response to IL-4 (45). The addition of 15-HETE, with its associated increase in 15-HETE-PE, also enhanced IL-13-induced MUC5AC expression. In the absence of IL-13, no increases in 15 HETE-PE and MUC5AC expression were observed. These results suggest that 15-HETE-PE enhances, rather than initiates, gene expression, likely through effects on IL-13-activated signaling pathways, including the ERK/ MAPK pathway as suggested by a previous study (46).

Inhibition of 15LO1 with PD146176 further confirmed the effect of this pathway on MUC5AC expression. PD146176, which decreased levels of 15-HETE-PE in our cell culture system, also significantly decreased IL-13-induced MUC5AC expression. However, because the specificity of this inhibitor for 15LO1 alone is not known, these results were further confirmed by transfection of the cells with a specific 15LO1 siRNA (ALOX15 siRNA). Similar to PD146176, ALOX15 siRNA transfection suppressed MUC5AC gene expression induced by IL-13, which was restored with the addition of exogenous 15-HETE. Although the siRNA inhibited 15LO1 mRNA and protein, it did not further decrease the 15-HETE-PE levels. Because the siRNA experiments must be performed in the early days of culture when the cells are proliferating (to achieve appropriate levels of transfection efficiency), it is conceivable that the impact of the siRNA on the low 15-HETE-PE levels cannot be discerned by LC/ESI/ MS. It is also possible that 15LO2, which is present in these cells and is not affected by IL-13 (*see* Figure 3D), could have

Figure 6. IL-13 induces MUC5AC expression in human bronchial epithelial cells *in vitro*. Primary human bronchial epithelial cells from 27 subjects (10 severe asthma, 6 mild-moderate asthma, and 11 normal control) were cultured with or without IL-13 (10 ng/ml) stimulation for 10 days. Relative mRNA expression levels were calculated using the delta Ct method.

Figure 7. Exogenous 15-HETE enhances MUC5AC expression induced by IL-13 *in vitro*. Primary human bronchial epithelial cells (1 severe asthma, 1 mild-moderate asthma, and 2 normal control) were cultured with or without IL-13 (10 ng/ml) stimulation for 10 days. Additional 15-HETE (1 μ M, unconjugated) was added to the medium for 24 or 72 hours before harvest. Ethanol (1%) was added to the medium as the vehicle control.

Figure 8. Pharmacologic inhibition of 15LO1 decreases IL-13-induced 15-HETE-PE conjugation and MUC5AC mRNA expression *in vitro*. Primary human bronchial epithelial cells were stimulated with IL-13 (10 ng/ml) for 10 days with or without the 15LO1 inhibitor (PD146176, 1 μ M). (A) The pharmacologic 15LO1 inhibitor (PD146176, 1 μ M) decreased the intracellular levels of 15-HETE-PE induced by IL-13 stimulation (n = 3, 2 severe asthma, and 1 normal control). (B) PD146176 also suppressed IL-13-induced MUC5AC mRNA expression (n = 8, 2 severe asthma, 2 mild-moderate asthma, and 4 normal control).

contributed to the majority of the 15-HETE-PE produced at this early stage of ALI.

The results from this study are in contrast to results from an earlier study that evaluated the role of 15LO1 in mucus secretion (22). In that system, neither IL-4/IL-13 nor 15-HETE altered mucin gene expression. The differences in the studies are likely due to the different experimental systems, including

the short exposure times to IL-4/IL-13. In that study, the stimulation of IL-4/IL-13 was performed during the early stages of ALI (<24 hours), when MUC5AC expression in response to Th2 stimulation was low. Our study similarly showed that 15-HETE alone could not induce MUC5AC expression. 15-HETE only enhances MUC5AC expression in the presence of IL-13 stimulation.

Figure 9. 15LO1 siRNA transfection suppressed IL-13-induced MUC5AC mRNA expression *in vitro*, and exogenous 15-HETE neutralized this suppression. Primary human bronchial epithelial cells were transiently transfected with 15LO1 siRNA (ALOX15 siRNA) and stimulated with IL-13 (10 ng/ml) for 2 days under acute lung injury. Unconjugated exogenous 15-HETE (1 μ M) was added to medium 24 hours before harvest. All values expressed as fold changes induced by IL-13 stimulation. ALOX15 siRNA transfection significantly suppressed IL-13-induced 15LO1 (A) mRNA and (B) protein expression *in vitro* (n = 5, 3 severe asthma, and 2 normal control). (C) ALOX15 siRNA transfection suppressed IL-13-induced MUC5AC mRNA expression and exogenous 15-HETE neutralized this suppression (n = 9, 5 severe asthma, 1 mild-moderate asthma, and 3 normal control).

As part of this study on the relationship of 15LO1 to MUC5AC expression, the spectrum of arachidonic acid substrates produced by 15LO1 was analyzed. In contrast to what has been reported in monocytes/macrophages (23), nearly all endogenous 15-HETE produced in response to IL-13 stimulation was esterified with PE, and no free 15-HETE was detected. This is the first report we are aware of showing formation of 15-HETE conjugated with PE in airway epithelial cells in response to IL-13 and an associated biologic function. Although 15LO2 could metabolize Arachidonic acid to generate 15-HETE, it is likely that the majority of 15-HETE-PE arose from 15LO1 because 15LO2 protein did not increase in response to IL-13. An increase in esterified 15-HETE also occurred in response to the addition of exogenous 15-HETE in the presence of IL-13 stimulation. Whether the esterification of 15-HETE to PE in response to IL-13 stimulation protects the 15-HETE from being metabolized into lipoxins (or other potentially antiinflammatory compounds) by other eicosanoid enzymes is not clear. However, it could explain the observation that, although total 15-HETE levels in asthma are high (with typical ELISAs not discriminating esterified from free 15-HETE), the downstream “antiinflammatory” metabolites—the lipoxins—are low (47, 48). Finally, although 15S-HETE-PE was the only eicosanoid identified in this primary human epithelial cell system before or after IL-13 stimulation (using exploratory but extensive GC/MS/LS technology), this may not exclude other eicosanoids from regulating MUC5AC expression *in vivo*. Further studies may reveal the involvement of other eicosanoids in the regulation of airway mucus production.

In summary, our present study with primary human airway epithelial cells from subjects with asthma and normal subjects indicates that 15LO1 and its primary metabolite, 15-HETE-PE, enhance IL-13-induced MUC5AC expression in human bronchial epithelial cells. Thus, the high levels of 15LO1 expression observed in asthmatic epithelial cells could contribute to the sustained increases in MUC5AC observed in this disease. Trials of 15LO1 pathway inhibitors in asthma could eventually be a promising treatment strategy.

Conflict of Interest Statement: J.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.B.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.E.W. has received consulting fees from GlaxoSmithKline and Wyeth (\$3,000 in 2006, \$5,000 in 2007, and \$3,000 in 2008 for GlaxoSmithKline and \$2,000 in 2008 from Wyeth). S.W.'s lab will receive grants from GlaxoSmithKline and Aerovance to do studies with IL-4/13 inhibitors, but the amount is not yet known.

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