

IL-13 desensitizes β_2 -adrenergic receptors in human airway epithelial cells through a 15-lipoxygenase/G protein receptor kinase 2 mechanism

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Background: β_2 -Adrenergic receptor (β_2 AR) agonists are critical treatments for asthma. However, receptor desensitization can lead to loss of therapeutic effects. Although desensitization to repeated use of β_2 -agonists is well studied, type 2 inflammation could also affect β_2 AR function.

Objective: We sought to evaluate the effect of the type 2 cytokine IL-13 on β_2 AR desensitization in human airway epithelial cells (HAECs) and determine whether 15-lipoxygenase-1 (15LO1) binding with phosphatidylethanolamine-binding protein 1 (PEBP1) contributes to desensitization through release of G protein receptor kinase 2 (GRK2).

Methods: HAECs in air-liquid interface culture with or without IL-13 (48 hours) or isoproterenol hydrochloride (ISO; 30 minutes) pretreatment were stimulated with ISO (10 minutes). Cyclic adenosine 3, 5-monophosphate (cAMP) levels were measured using ELISA, and β_2 AR and GRK2 phosphorylation was measured using Western blotting. Short interfering RNA was used for 15LO1 knockdown. Interactions of GRK2, PEBP1, and 15LO1 were detected by means of immunoprecipitation/Western blotting and immunofluorescence. HAECs and airway tissue from control subjects and asthmatic patients were evaluated for 15LO1, PEBP1, and GRK2.

Results: Pretreatment with ISO or IL-13 decreased ISO-induced cAMP generation compared with ISO for 10 minutes alone paralleled by increases in β_2 AR and GRK2

phosphorylation. GRK2 associated with PEBP1 after 10 minutes of ISO in association with low phosphorylated GRK2 (pGRK2) levels. In contrast, in the presence of IL-13 plus ISO (10 minutes), binding of GRK2 to PEBP1 decreased, whereas 15LO1 binding and pGRK2 levels increased. 15LO1 knockdown restored ISO-induced cAMP generation. These findings were recapitulated in freshly brushed HAECs from cells and tissue of asthmatic patients.

Conclusion: IL-13 treatment of HAECs leads to β_2 AR desensitization, which involves 15LO1/PEBP1 interactions to free GRK2, and allows it to phosphorylate (and desensitize) β_2 ARs, suggesting that the beneficial effects of β_2 -agonists could be blunted in patients with type 2 associated asthma. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: IL-13, cAMP, phosphatidylethanolamine binding protein 1, 15-lipoxygenase 1, β_2 -adrenergic receptor, G protein receptor kinase 2, asthma

β_2 -Agonist drugs, which are central to obstructive airway disease management, work through activation of G protein-coupled receptor (GPCRs), specifically the β_2 -adrenergic receptor (β_2 AR). This receptor is present on many cells, including airway epithelial cells and smooth muscle cells. Activation of β_2 ARs on airway smooth muscle leads to muscle relaxation and bronchodilation, whereas acute epithelial cell effects include enhanced ciliary beat and mucus clearance.¹ Chronic exposure of a GPCR to its ligand leads to receptor desensitization through receptor phosphorylation and internalization. Indeed, continuous β_2 -agonist use results in loss of bronchodilator and bronchoprotective effects²⁻⁷ and deterioration of asthma control.^{8,9} After ligand stimulation and arrestin binding, β_2 ARs are internalized. They can then be recycled to the surface after removal of ligand or more permanently made inaccessible in states of chronic stimulation.^{10,11} GPCR kinases, specifically G protein receptor kinase 2 (GRK2), play important roles in mediating β_2 AR desensitization.^{12,13} GRK2 phosphorylates the receptor, allowing arrestins to bind, effectively blocking receptor interaction and activation of heterotrimeric G proteins.¹⁴

Interestingly, GRK2 also interacts with the multifunctional protein phosphatidylethanolamine-binding protein 1 (PEBP1). Normally, PEBP1 binds to and inactivates Raf-1 (an activator of extracellular signal-regulated kinase [ERK] pathways), thereby serving as an endogenous inhibitor of those pathways.¹⁵⁻¹⁹ Under inflammatory conditions that phosphorylate PEBP1, conformational changes in PEBP1 induce its dissociation from Raf-1, allowing PEBP1 to bind GRK2.^{20,21} This binding to PEBP1 prevents GRK2 from phosphorylating the β_2 AR, allowing ongoing receptor activation.

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Abbreviations used

ALI:	Air-liquid interface
ALOX15:	Arachidonate 15-lipoxygenase
β2AR:	β2-Adrenergic receptor
cAMP:	Cyclic adenosine 3, 5-monophosphate
ERK:	Extracellular signal-regulated kinase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GCV:	Geometric coefficient of variation
GPCR:	G protein-coupled receptor
GRK2:	G protein receptor kinase 2
HAEC:	Primary human airway epithelial cell
HC:	Healthy control subject
ICS:	Inhaled corticosteroid
IF:	Immunofluorescence
IP:	Immunoprecipitation
ISO:	Isoproterenol hydrochloride
LABA:	Long-acting β2-agonist
15LO1:	15-Lipoxygenase-1
pβAR2:	Phosphorylated β2AR
PEBP1:	Phosphatidylethanolamine-binding protein 1
pGRK2:	Phosphorylated GRK2
SABA:	Short-acting β-agonist
siRNA:	Short interfering RNA

Recent studies showed that 15-lipoxygenase-1 (15LO1), an enzyme induced by the type 2/T_H2 cytokine IL-13 in epithelial cells, also binds to PEBP1. 15LO1 is upregulated in asthmatic patients in association with IL-4/IL-13/type 2 inflammation, ERK activation, and increases in levels of the mucin MUC5AC.^{22,23} Importantly, similar to GRK2, enhanced 15LO1 expression by IL-13 displaces Raf-1 from PEBP1, allowing it to bind 15LO1, an association recapitulated in epithelial cells from asthmatic patients *in vivo*. *In vitro* this binding enhances ERK activation and MUC5AC expression.

Therefore we hypothesized that under type 2 conditions, as evidenced by the high 15LO1 levels seen in approximately 50% of asthmatic patients, 15LO1 would preferentially bind to PEBP1 while displacing (and freeing up) GRK2 to be phosphorylated. This phosphorylated GRK2 would phosphorylate and desensitize the β2AR, decreasing cyclic adenosine 3, 5-monophosphate (cAMP) response to β-agonists. Primary human airway epithelial cells (HAECs) were evaluated for their cAMP response to β2AR stimulation, as well as their levels of phosphorylated β2AR, in the presence and absence of IL-13-induced 15LO1 to address this hypothesis. The role of IL-13-induced 15LO1 was evaluated by measuring the binding of 15LO1 and GRK2 to PEBP1 *in vitro*. The subsequent effect of 15LO1 knockdown on β2AR desensitization/cAMP activation was then addressed. These *in vitro* experiments were compared to *in vivo* findings in airway epithelial cells and tissues from asthmatic patients and control subjects.

METHODS**Study participants**

Asthmatic participants met American Thoracic Society criteria for asthma²⁴ and were recruited as part of the National Heart, Lung, and Blood Institute's Severe Asthma Research Program (National Institutes of Health grant AI-40600) or the Electrophilic Fatty Acid Derivatives in Asthma studies.²⁵ Patients with mild-to-moderate asthma had an FEV₁ of 60% of predicted value or greater on short-acting β-agonists (SABAs) only or on low-to

moderate-dose inhaled corticosteroids (ICSs; <880 μg/d fluticasone propionate or equivalent) plus SABAs. Severe asthma was defined by the American Thoracic Society 2000 definition, consisting of 1 or 2 major criteria (high-dose ICS, frequent use of oral corticosteroid, or both) with at least 2 of 7 minor criteria.²⁶ Healthy control subjects (HCs) had no history of respiratory disease or recent respiratory tract infection and normal lung function. No subject smoked within the last year or for greater than 5 pack years. The study was approved by the University of Pittsburgh Institutional Review Board, and all participants provided informed consent.

Bronchoscopy with epithelial airway brushings

Bronchoscopy with epithelial brushing was performed, as previously described.^{27,28} Per protocol, all participants, including HCs, received 2.5 mg of nebulized albuterol within 15 to 45 minutes of the procedure.

Primary HAEC culture and short interfering RNA transfection

Primary HAECs were cultured in air-liquid interface (ALI) cultures, as previously described.²⁸⁻³⁰ Short interfering RNA (siRNA) transfection was performed on 70% confluent cells in 12-well transwell plates during submerged culture by using Mirus si-QUEST transfection reagent (Mirus Bio, Madison, Wis). For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

Western blotting

Cell lysates were run on 4-12% SDS-PAGE gels under reducing conditions, as previously described.²⁸

cAMP assay

Cells were washed with cold PBS, stimulated for 10 minutes with 1 μmol/L isoproterenol hydrochloride (ISO), and lysed for cAMP levels by using a competitive ELISA kit.

Coimmunoprecipitation

Cells were lysed, precleaned with Protein A agarose beads (Santa Cruz, Calif) and incubated with pull-down antibody before incubation with Protein A agarose beads. Immunoprecipitates were centrifuged and separated on 4-12% SDS/PAGE gels for Western blotting by using primary antibodies generated from different species from the pull-down antibody. For details, see the Methods section in this article's Online Repository.

Immunofluorescence and confocal microscopy

Tissues were fixed in acetone and embedded in glycol-methacrylate for immunofluorescence (IF) staining, as previously described.²⁹ For details, see the Methods section in this article's Online Repository.

Statistical analysis

Clinical demographics data were generally normally distributed and analyzed using the *t* test; categorical data were analyzed using χ² analysis. All subjects were divided into 15LO1 Lo versus Hi groups on the basis of the median split of the 15LO1 protein expression data in fresh epithelial cells. Clinical experimental data were not normally distributed, but the majority of fresh epithelial cell data were normalized by means of natural log transformation and could be analyzed with the *t* test. *Ex vivo* coimmunoprecipitation data were not normally distributed after log transformation and were therefore analyzed nonparametrically with Wilcoxon rank sum testing.

In vitro data, including percentage/fold changes, were analyzed using linear and nonlinear models. All control and stimulated condition values were normally distributed, except for IL-13-stimulated phosphorylated GRK2 (pGRK2), whereas the fold changes were normally distributed using natural

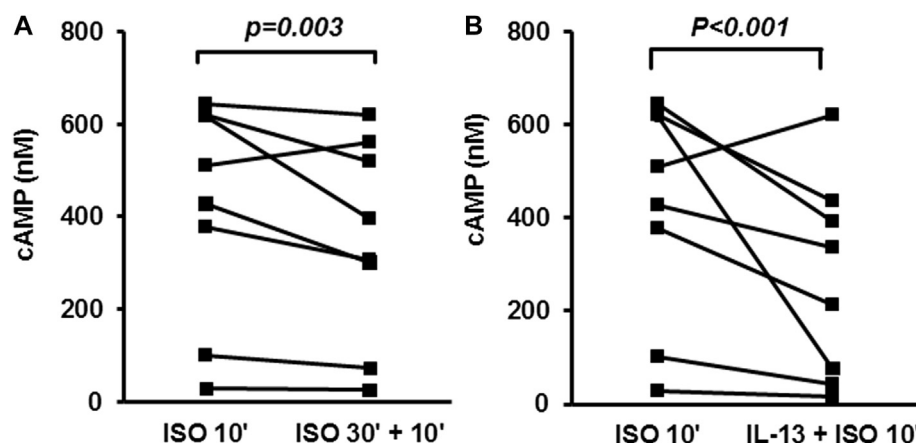


FIG 1. IL-13 pretreatment decreases cAMP release in response to isoproterenol stimulation. ALI-cultured primary bronchial airway epithelial cells were pretreated with either ISO (30 minutes) or IL-13 (48 hours), followed by stimulation with ISO for 10 minutes. Cells were harvested for cAMP levels by means of ELISA. **A**, ISO stimulation for 10 minutes induced high cAMP levels (415 ± 84 [SEM]nM), which were inhibited by ISO (30 minutes) pretreatment (350 ± 77 [SEM]nM). **B**, IL-13 pretreatment for 48 hours inhibited cAMP release induced by ISO for 10 minutes alone (267 ± 76 [SEM]nM).

log transformation. The natural log of the fold change was compared with zero using normal approximation for null distribution to test the differences between the control and stimulated groups. Although analytically we used means and SDs (p β 2AR and pGRK2) or SEMs (cAMP) of log-transformed data, we reported the results as the geometric mean and geometric coefficient of variation (GCV). Comparisons of fold changes (IL-13 vs ISO30 pretreatment) using the *t* test were performed on normally distributed data. No formal adjustment for multiple testing was applied. Statistical analysis was performed with JMP SAS software (SAS Institute, Cary, NC). *P* values of less than .05 were considered statistically significant.

RESULTS

In vitro studies

Subjects' demographics. Airway epithelial cells were obtained from a total of 73 subjects, including 15 HCs and 58 asthmatic patients. *In vitro* culture studies were performed on HAECs from 5 HCs and 44 asthmatic patients (see Table E1 in this article's Online Repository at www.jacionline.org). There were no differences in responses by asthma disease state or severity.

IL-13 decreases cAMP generation in response to isoproterenol. cAMP generation was evaluated in response to ISO ($1 \mu\text{mol/L}$ for 10 minutes) in the presence or absence of IL-13 (10 ng/mL) for 48 hours and compared with the cAMP response to a second addition of ISO after 30 minutes of ISO ($1 \mu\text{mol/L}$) before treatment, a protocol that reduces cAMP response, to determine whether IL-13 affects β 2AR desensitization in HAECs.³¹ Stimulation of cells with ISO for 10 minutes induced a high level of cAMP release (Fig 1). There was a 17% (GCV = 12%) decrease in cAMP by ISO pretreatment for 30 minutes ($P = .003$; Fig 1, A). IL-13 pretreatment for 48 hours inhibited cAMP release 46% (GCV = 25%) in response to ISO (10 minutes, $P < .001$; Fig 1, B). Although not statistically significant, IL-13 tended to reduce ISO-induced cAMP release to a greater degree than prior ISO treatment ($P = .066$, data not shown).

IL-13 and ISO induce phosphorylation of β 2AR and GRK2. Because IL-13 pretreatment inhibited cAMP generation in response to ISO, HAECs were treated with either

IL-13 (10 ng/mL) for 48 hours or ISO ($1 \mu\text{mol/L}$) for 30 minutes, and β 2AR phosphorylation was measured and indexed to total β 2AR. Both IL-13 and ISO increased phosphorylated β 2AR (p β 2AR) compared with medium alone (54% [GCV = 32%] increase [$P = .022$] and 45% [GCV = 25%] increase [$P < .05$], respectively; Fig 2, A, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). Similar increases were observed when p β 2AR was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; $P = .02$; see Fig E1, B and D). There were no effects of IL-13 on total β 2AR protein expression or membrane translocation. However, IL-13 did increase cytosolic p β 2AR levels (see Fig E1, B, C, and E). Increases in p β 2AR levels paralleled decreases in cAMP levels after pretreatment with IL-13 or ISO.

Previous studies suggest phosphorylation of GRK2 is required for β 2AR phosphorylation and desensitization.³² GRK2 phosphorylation was evaluated in the presence and absence of IL-13 and ISO pretreatment by means of Western blotting. GAPDH was applied as the loading control (see the Methods section in this article's Online Repository). ISO marginally increased GRK2 phosphorylation by 35% (GCV = 18%, $P = .07$), whereas IL-13 increased pGRK2 levels by 58% (GCV = 20%, $P < .05$; Fig 2, B, and see Fig E2 in this article's Online Repository at www.jacionline.org), suggesting IL-13 promotes β 2AR phosphorylation and desensitization through a GRK2 mechanism.

IL-13 suppresses GRK2 binding to PEBP1 in response to ISO *in vitro*: Potential involvement of 15LO1 in β 2AR phosphorylation and desensitization. β 2AR activation is regulated by interactions of GRK2 with PEBP1.^{16,17} Factors that prevent GRK2 binding to PEBP1 will increase GRK2 association with the β 2AR complex promoting receptor phosphorylation and desensitization.²² We hypothesized that under IL-13/type 2 conditions, 15LO1 would displace GRK2 from PEBP1, allowing it to phosphorylate the β 2AR. Accordingly, primary HAECs were treated with IL-13 plus ISO or ISO alone, and immunoprecipitation (IP)/Western blotting or IF staining was performed. As expected, GRK2 bound with PEBP1 in response to ISO (Fig 3, A). In contrast, IL-13 (10 ng/mL) pretreatment decreased binding of GRK2 to PEBP1 in response to ISO compared with that seen after

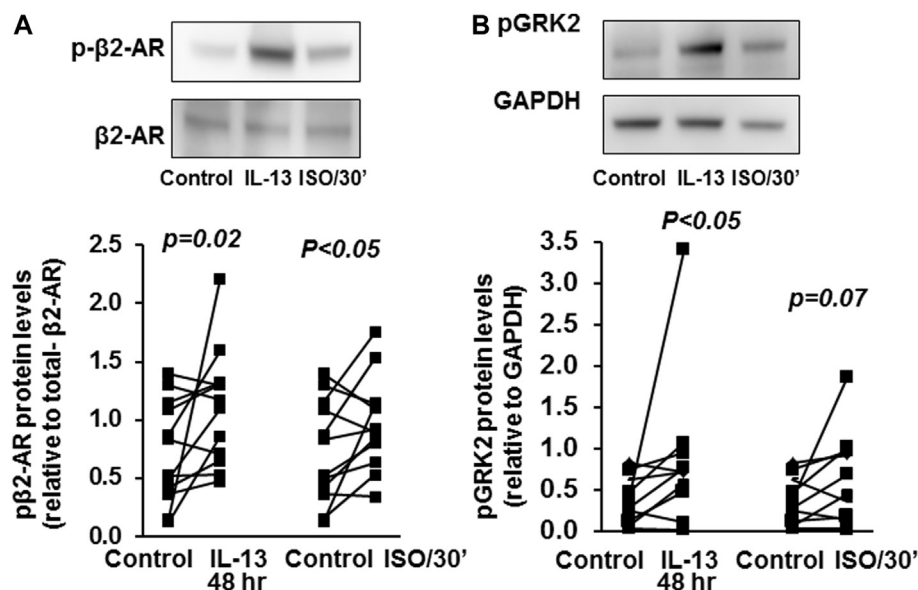


FIG 2. IL-13 and ISO induce β2AR and GRK2 phosphorylation in primary human bronchial airway epithelial cells. ALI-cultured bronchial airway epithelial cells were stimulated with ISO (30 minutes) or IL-13 (48 hours). Total protein was harvested for β2AR and GRK2 phosphorylation by means of Western blotting and densitometric analysis. **A**, Both IL-13 (1.06 ± 0.13) and ISO (1.01 ± 0.13) significantly increased β2AR phosphorylation compared with medium alone (0.75 ± 0.11). **B**, Both IL-13 (0.88 ± 0.27) and ISO (0.69 ± 0.16) significantly increased GRK2 phosphorylation compared with medium alone (0.39 ± 0.08).

ISO alone ($P = .032$; Fig 3, A and B, upper panel), whereas as expected (and in contrast), the binding of PEBP1 with 15LO1 increased ($P = .048$; Fig 3, B, lower panel).

Confocal analysis confirmed these studies. ISO (10 minutes) increased colocalization of GRK2 with PEBP1 compared with control values, whereas in the presence of IL-13, this colocalization was suppressed (Fig 3, C, upper panel). In contrast, 15LO1 was highly bound to PEBP1 in the presence of IL-13 and not affected by ISO (Fig 3, C, lower panel). Thus IL-13-induced 15LO1 competitively binds with PEBP1, decreasing GRK2 binding to PEBP1 *in vitro*.

Knockdown of 15LO1 in the presence of IL-13 restores cAMP generation in response to ISO stimulation. To confirm that high 15LO1 levels inhibit cAMP generation, siRNA knockdown of 15LO1 in the presence of IL-13 was performed, and cAMP and pβ2AR levels were measured. IL-13 induced 15LO1 protein expression ($P = .013$), although ISO (30 minutes) did not ($P = .4$, see Fig E3 in this article's Online Repository at www.jacionline.org). Arachidonate 15-lipoxygenase (ALOX15) siRNA knocked down 15LO1 protein by 30% to 80% (Fig 4, A, and see Fig E4, A and B, in this article's Online Repository at www.jacionline.org). ALOX15 siRNA transfection also decreased β2AR phosphorylation by 63% to 86% in the presence of IL-13 plus ISO (10 minutes, $P < .001$; Fig 4, A). ALOX15 knockdown in IL-13-stimulated cells increased cAMP generation by 34% ($GCV = 35\%$) in response to ISO (10 minutes) compared with scrambled siRNA ($P = .017$; Fig 4, B) and as fold change compared with scrambled fold change ($P = .02$, see Fig E4, C). No changes were observed in total β2AR after ALOX15 siRNA transfection (Fig 4, A, and see Fig E4, A and D). The decrease in β2AR phosphorylation and restoration of cAMP release after ALOX15 knockdown confirmed that 15LO1 desensitizes β2AR, likely through a GRK2/PEBP1 mechanism.

Ex vivo studies

Subjects' demographics. *Ex vivo* studies of freshly brushed epithelial cells (Western blotting) were performed on 36 subjects, including 10 HCs and 26 asthmatic patients (Table I). Subjects were categorized by 15LO1 phenotype (Lo vs Hi as median split). 15LO1 Hi subjects were more likely to be corticosteroid-naïve patients with mild asthma, or patients with severe asthma, the majority of whom were receiving oral corticosteroids and were more likely to be male. There were no differences in FEV₁, SABA, or long-acting β₂-agonist (LABA) use between the 2 phenotypes. However, fractional exhaled nitric oxide levels were significantly greater in 15LO1 Hi subjects compared with those seen in 15LO1 Lo subjects ($P < .001$). Fresh HAECs from 14 subjects were used for IP/Western blotting (Table II). In small numbers there were no differences in 15LO1 Lo versus Hi subjects, except age was greater in the 15LO1 Hi group.

β2AR and GRK2 phosphorylation positively correlates with 15LO1 expression in HAECs *ex vivo* (Table II). Because *in vitro* data suggested a relationship of pGRK2 to pβ2AR (and receptor desensitization), the relation of pGRK2 to pβ2AR was evaluated by means of Western blotting *ex vivo* in freshly brushed airway epithelial cells (see the Methods section in this article's Online Repository). Although absolute levels varied within subjects, perhaps because of albuterol pretreatment of all participants before bronchoscopy, pβ2AR levels strongly correlated with pGRK2 levels in these cells ($n = 36$, $R = 0.87$, $P < .001$; Fig 5, A and B). pβ2AR and pGRK2 levels also positively correlated with 15LO1 expression ($r = 0.52$ and 0.47 and $P = .002$ and $.005$, respectively; data not shown). When subjects were categorized into 15LO1 Lo and 15LO1 Hi groups by using a median split of 15LO1 protein data, pGRK2 and pβ2AR levels were significantly greater in the 15LO1 Hi group than in the 15LO1 Lo group (Fig 5, C: $P = .02$; Fig 5, D: $P = .04$,

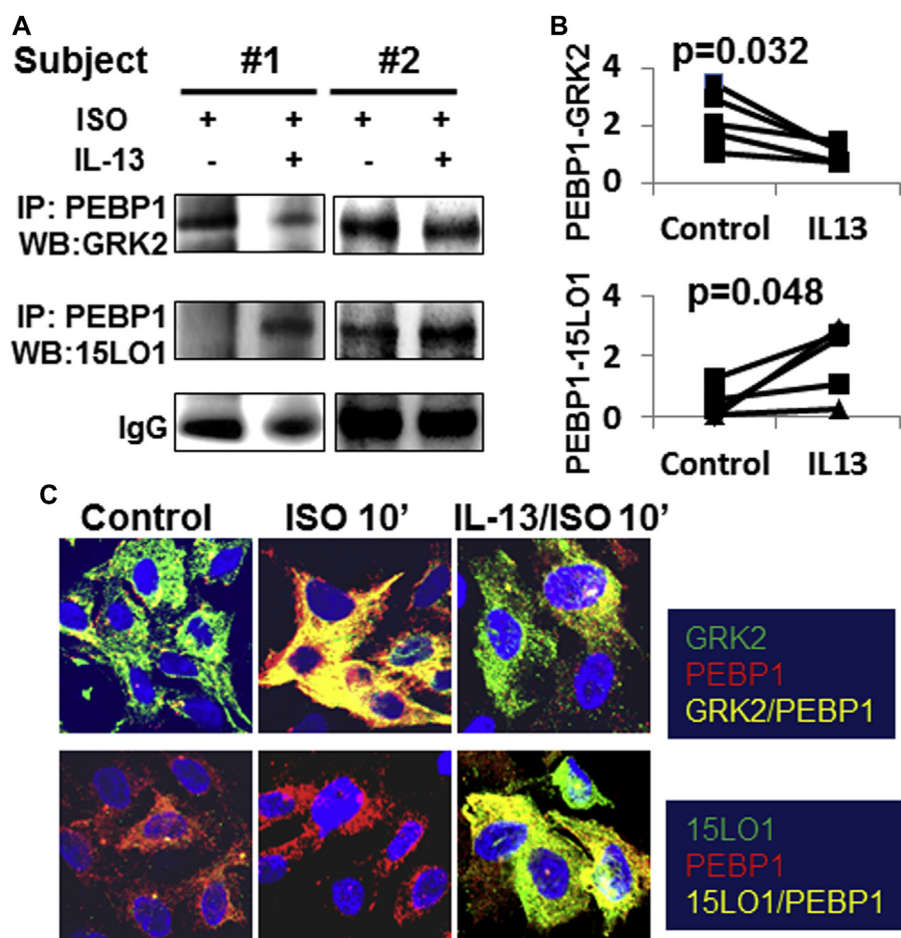


FIG 3. IL-13 suppresses GRK2 binding to PEBP1 and enhances 15LO1 binding to PEBP1 in response to ISO *in vitro*. Bronchial airway epithelial cells in the presence or absence of IL-13 (48 hours) were stimulated with ISO for 10 minutes. Total protein was harvested for Western blotting (WB)/IP. Cells were also fixed for IF staining. **A**, GRK2 bound with PEBP1 in response to ISO, whereas pretreatment with IL-13 markedly decreased binding of GRK2 to PEBP1 and increased 15LO1 binding to PEBP1. **B**, Densitometric analysis of Western blotting/IP. **C**, Confocal analysis showing that ISO treatment increased the colocalization (yellow) of GRK2 (green) with PEBP1 (red) compared with control values. This colocalization was less in the presence of IL-13. In contrast, 15LO1 (green) was highly expressed and colocalized/bound (yellow) to PEBP1 in the presence of IL-13 and not affected by ISO.

respectively). No change in total GRK2 and β 2AR protein expression was seen across the subjects *ex vivo* (see Fig E5 in this article's Online Repository at www.jacionline.org).

15LO1 binding to PEBP1 is associated with less GRK2 binding to PEBP1 in cells and tissue from asthmatic patients (Table II). Because *in vitro* data suggested that IL-13-induced 15LO1 competitively binds PEBP1 to decrease its binding to GRK2, this association was also evaluated *ex vivo* in freshly brushed airway epithelial cells by means of IP/Western blotting. As noted, before bronchoscopy, all participants received albuterol as a safety measure, which could increase GRK2 binding to PEBP1 in the absence of 15LO1. Although levels of GRK2 binding to PEBP1 varied across subjects (Fig 6, A and B, and Table II), in general, higher levels of 15LO1 binding to PEBP1 were associated with lower GRK2 binding. These increases in 15LO1 binding to PEBP1 paralleled the 15LO1 levels observed by means of Western blotting.

IF/confocal microscopy was performed on lung tissue from 8 asthmatic patients and 8 HCs to support this *ex vivo* observation

further (subjects' characteristics are shown in Table E2 in this article's Online Repository at www.jacionline.org). As expected, 15LO1 colocalization with PEBP1 was higher in most asthmatic patients compared with that seen in HCs (Fig 6, C, representative image). In contrast, GRK2 colocalization with PEBP1 in the airway epithelium was lower in asthmatic patients compared with HCs, which is consistent with the IP/Western blotting, suggesting 15LO1 binding to PEBP1 can increase free/phosphorylated GRK2, which then phosphorylates and desensitizes the β 2AR in the presence of IL-13.

DISCUSSION

β ₂-Agonists are indispensable asthma treatments, primarily inducing bronchodilation through effects on smooth muscle.³³ However, their effects on epithelial cells to clear mucus are also important.³⁴ This study is the first to observe a clinically meaningful effect of IL-13 to decrease β -agonist responsiveness in primary HAECs and to identify complex interactions between

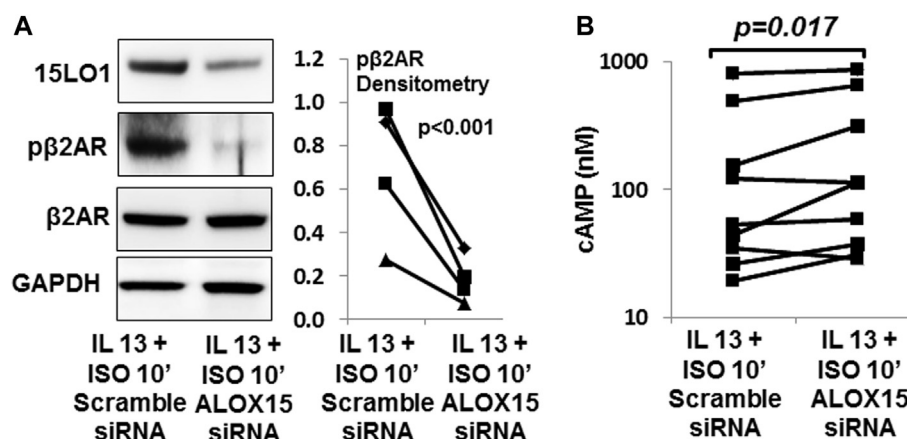


FIG 4. ALOX15 siRNA knockdown of 15LO1 expression restored ISO-induced cAMP generation in bronchial airway epithelial cells. ALI-cultured cells transfected with ALOX15 siRNA or scrambled siRNA were stimulated with IL-13 for 48 hours plus ISO for 10 minutes. Total protein was harvested for Western blotting and ELISA analysis. Data are presented as means of duplicates. **A**, ALOX15 siRNA knocked down 15LO1 protein and decreased β2AR phosphorylation induced by IL-13/ISO stimulation. **B**, ALOX15 siRNA knockdown restored ISO (10 minutes)-induced cAMP generation in cells treated with IL-13 for 48 hours (244 ± 102 [SEM]nM) compared with scrambled siRNA (196 ± 91 [SEM]nM).

TABLE I. Characteristics of subjects undergoing analysis of fresh epithelial cells (n = 36)

	15LO1 Lo*	15LO1 Hi	P value
All subjects			
Age (y), mean ± SD	40 ± 12	39 ± 1	.8
Sex (% female)	82	42	.01
Race (% [W/AA/other])	65/29/6	79/19/8	.34
FEV ₁ (% predicted), mean ± SEM	88 ± 7	77 ± 5	.18
Bronchodilator response (%), mean ± SEM	12 ± 3	16 ± 4	.31
Severity† (%)	29/18/29/24	26/26/0/47	.03
FENO (ppb), mean ± SEM	21 ± 3	45 ± 7	.007
Asthmatic patients only (n = 26)			
ICS (% [no/yes])	17/83	36/64	.27
LABA (% [no/yes])	42/58	57/43	.43
SABA (puffs/wk), mean ± SD	6 ± 8	15 ± 15	.09

AA, African American; FENO, fraction of exhaled nitric oxide; W, white.

*15 LO1 Lo/Hi was calculated as the median split of densitometric data for 15LO1 among all subjects.

†Severity = HCs/patients with mild asthma but no ICS use/patients with mild-to-moderate asthma + ICS use/patients with severe asthma.

15LO1, GRK2, and PEBP1 as contributing to this effect. In cultured human HAECs IL-13 induces GRK2 and β2AR phosphorylation while promoting 15LO1 expression. Enhanced 15LO1 expression contributes to increases in pGRK2 levels by competitively binding to PEBP1, freeing GRK2 from its binding to PEBP1. Free GRK2 can then phosphorylate β2AR, desensitizing it and decreasing downstream activation, as measured by lower cAMP production. These *in vitro* interactions were present in epithelial cells and tissue *ex vivo* in asthmatic patients. Importantly, siRNA knockdown of 15LO1 restored cAMP generation in response to isoproterenol *in vitro*. These results strongly suggest that the increased 15LO1 expression observed in asthmatic patients, particularly in relation to type 2 inflammation, contributes to desensitization of the β2AR in HAECs.

β2AR belongs to the 7-transmembrane GPCR family. β2AR activation enhances adenylate cyclase activity and cAMP

TABLE II. Characteristics of subjects for coimmunoprecipitation analysis (n = 14)

	15LO1 Lo*	15LO1 Hi	P value
All subjects			
Age (y), mean ± SD	38 ± 4	54 ± 4	.01
Sex (% female)	38	33	.8
Race (% [W/AA/other])	86/14/0	50/50/0	.14
FEV ₁ (% predicted), mean ± SEM	80 ± 7	67 ± 9	.3
Bronchodilator response (%), mean ± SEM	7 ± 3	5 ± 3	.59
Severity† (%)	37/13/13/37	17/17/33/33	.73
FENO (ppb), mean ± SEM	37 ± 10	34 ± 11	.8
Asthmatic patients only (n = 10)			
ICS (% [no/yes])	20/80	20/80	1.0
LABA (% [no/yes])	40/60	20/80	.29
SABA (puffs/wk), mean ± SD	10 ± 4	6 ± 4	.4

AA, African American; FENO, fraction of exhaled nitric oxide; W, white.

*15LO1 Lo/Hi was calculated as the median split of densitometric data for 15LO1 among all subjects.

†Severity = HCs/patients with mild asthma but no ICS use/patients with mild-to-moderate asthma + ICS use/patients with severe asthma.

synthesis, which promote smooth muscle relaxation in airways and blood vessels and enhance ciliary beating, thus enhancing mucin clearance.^{35,36} However, repeated β2AR stimulation leads to receptor desensitization through phosphorylation of GRK2, which promotes phosphorylation and internalization of β2AR.^{12,37} This receptor activation/desensitization is complex, with different mechanisms involved in different settings, although phosphorylation of β2AR is central to all.³⁴ Although inflammation is likely to play a role in modulating β2AR desensitization, it has not been extensively studied. However, anti-inflammatory drugs, such as corticosteroids, have been reported to reverse or prevent it,³⁸ whereas allergen challenge has been reported to slightly decrease β2AR sensitivity.³⁹

IL-13, a type 2 cytokine, plays a role in approximately 50% of asthmatic patients.⁴⁰ IL-13 blockade, alone or in combination with IL-4, through targeted biologic approaches, consistently improves outcomes in asthmatic patients with evidence for type 2

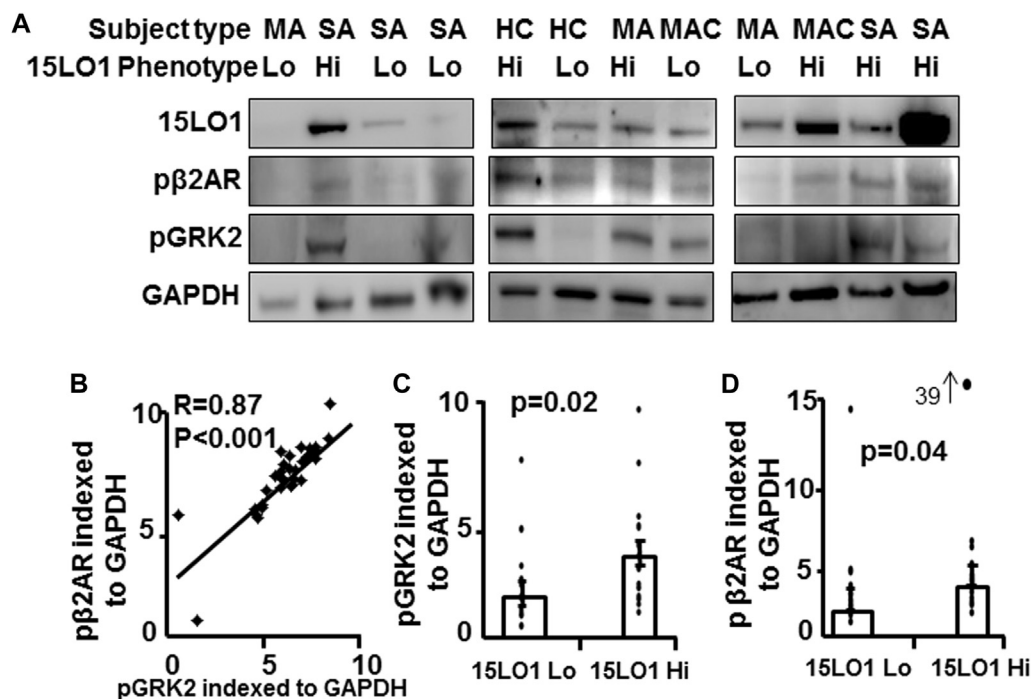


FIG 5. β2AR and GRK2 phosphorylation positively correlate with 15LO1 expression in HAECs *ex vivo*. **A**, Total protein from freshly brushed airway epithelial cells was harvested, and pβ2AR and pGRK2 levels were measured by means of Western blotting. Densitometric analysis was performed. pβ2AR, pGRK2, and 15LO1 were normalized to GAPDH and reported as arbitrary units. **B**, pβ2AR levels positively correlated with pGRK2 levels. **C** and **D**, pGRK2 (Fig 5, C) and pβ2AR (Fig 5, D) levels in the 15LO1 Hi group were higher compared with those in the 15LO1 Lo group. MA, Mild asthma/no ICS; MAC, mild-to-moderate asthma plus ICS; SA, severe asthma.

inflammation, with some of the most robust improvement seen in FEV₁, even when added to ICS/LABA therapy.^{41,42} This suggests that improvement in FEV₁ with these agents (on top of LABAs) might be due to an improved effect of the LABA. Although some of this effect is likely on airway smooth muscle responses, chronic improvement in FEV₁ in patients with more severe asthma likely also involves improved mucus clearance. Interestingly, another recent study suggests that tachyphylactic responses to β₂-agonists during exercise challenge might be related to the level of background type 2 immune processes because they appear more likely to occur in patients with high levels of fractional exhaled nitric oxide, a known IL-4/IL-13 biomarker.⁴³ Additionally, the β2AR gene is nested in the 5q31 region with the IL-4, IL-13, and IL-5 genes, suggesting common origins. However, their interactions have not been specifically explored. The results reported here specifically link IL-13 and β2AR function, with the presence of a type 2/IL-13 environment, leading to a nearly 50% reduction in cAMP response to β₂-agonist stimulation in primary HAECs. This reduced cAMP response associated with increased GRK2 and β2AR phosphorylation suggests IL-13 promoted β2AR desensitization through a GRK2 mechanism.³²

This association of IL-13-induced β2AR desensitization with GRK2 phosphorylation suggested that PEBP1 might be involved because under certain stimulatory conditions (protein kinase C activation and acute β₂-agonist stimulation), PEBP1 dissociates from Raf-1 and binds with GRK2.¹⁶ GRK2 binding to PEBP1 prevents it from phosphorylating β2AR, prolonging receptor activation and promoting activation of ERK through release of Raf-1 from PEBP1. However, we reported that in addition to

GRK2 and Raf-1 binding to PEBP1, the eicosanoid-generating enzyme 15LO1 also binds PEBP1.²⁹ 15LO1, a lipoxygenase that generates 15-hydroxyeicosatetraenoic acid, is upregulated in response to IL-13 stimulation of HAECs and is increased in asthmatic patients in relation to type 2/eosinophilic processes.^{23,27-29,44} In addition to upregulation of 15LO1 in response to IL-13, stimulation of HAECs with IL-13 promotes binding of 15LO1 to PEBP1, with subsequent release of Raf-1, activation of ERK, and increased expression of the airway mucin MUC5AC.²³ Thus we hypothesized that in the presence of IL-13, 15LO1 would compete with GRK2 for binding to PEBP1, leading to more free GRK2 and greater likelihood for β2AR desensitization (even in the absence of β₂-agonist stimulation).

As expected, GRK2 binding to PEBP1 was observed in cells stimulated with ISO for 10 minutes, supporting an active β2AR manifested by high cAMP expression. More importantly, pretreatment of cells with IL-13 decreased the association of GRK2 with PEBP1 in response to ISO and in association with increases in 15LO1 binding to PEBP1. This decrease in GRK2 binding to PEBP1 in the presence of IL-13 was associated with increased pGRK2 and pβ2AR levels and decreased cAMP production after ISO. Finally, 15LO1 knockdown prevented its interaction with PEBP1 but promoted binding of GRK2 to PEBP1, which returned production of cAMP after ISO to near baseline values.

To determine the relevance of these interactions to human disease, we evaluated the relationships of GRK2 and 15LO1 to PEBP1 in bronchial epithelial cells from asthmatic patients and

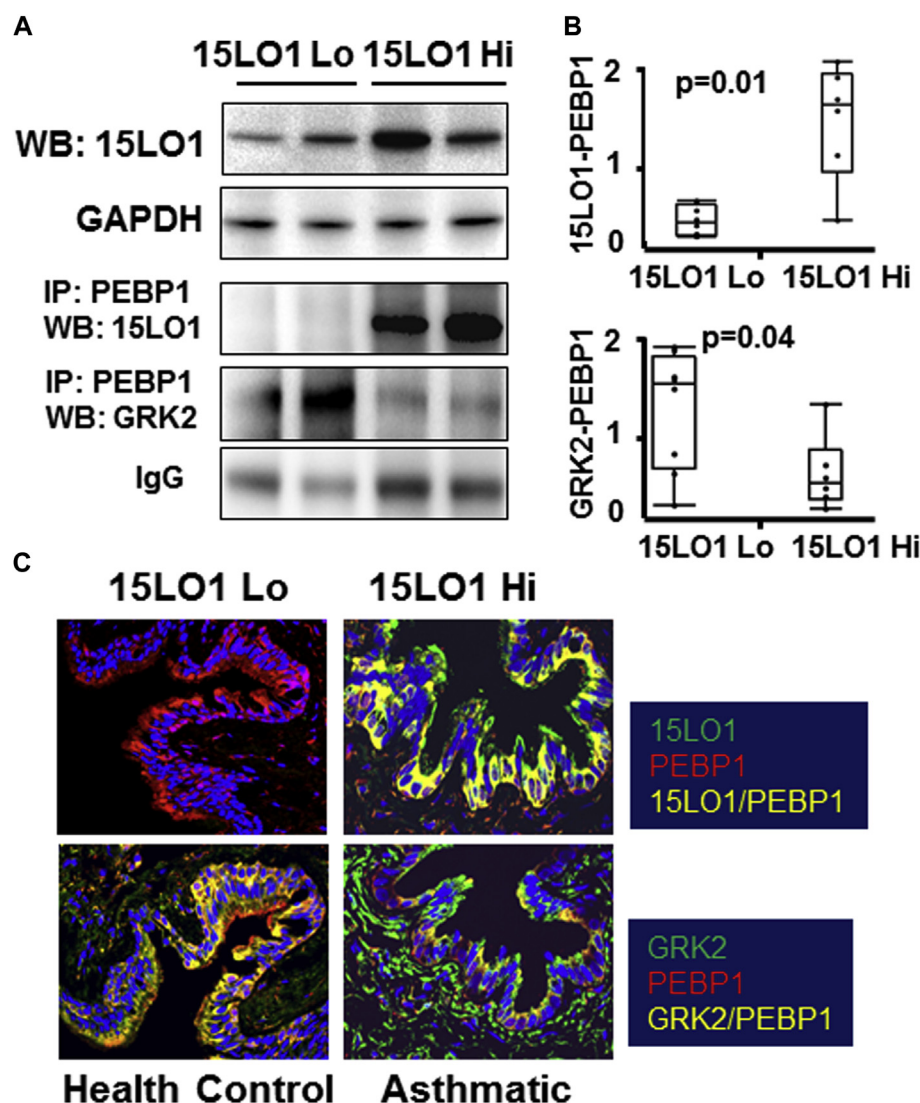


FIG 6. 15LO1 binding to PEBP1 is associated with less GRK2 binding to PEBP1 in cells and tissue from asthmatic patients compared with those from healthy control subjects. Total protein from freshly brushed airway epithelial cells was harvested for Western blotting (WB)/IP analysis. Lung tissue from asthmatic patients and HCs was fixed for immunofluorescent confocal microscopy studies. **A**, Higher levels of 15LO1 binding to PEBP1 were associated with lower GRK2 binding in freshly brushed epithelial cells. **B**, Densitometric analysis of Western blotting/IP. **C**, Expression of 15LO1 and its colocalization with PEBP1 were higher in an asthmatic patient compared with values in the control donor lung. In contrast, GRK2 colocalization with PEBP1 in the airway epithelium was lower in the asthmatic patient compared with an HC.

control subjects obtained using bronchoscopy. As noted, bronchoscopic samples were obtained after albuterol for safety reasons, which likely also promoted β_2 AR phosphorylation. Nevertheless, in the *ex vivo* samples greater 15LO1 expression was associated with greater pGRK2 and p β_2 AR levels. We also evaluated the binding of 15LO1 and GRK2 to PEBP1 in fresh epithelial cells using coimmunoprecipitation and Western blotting, as well as confocal microscopy on airway tissue. The coimmunoprecipitation studies demonstrated a strong inverse relationship of GRK2 binding to PEBP1 when compared with 15LO1 binding. Finally, the same associations were observed using confocal microscopy on tissue from asthmatic patients compared with that from control subjects. Thus the *in vitro* findings were recapitulated *ex vivo* in human asthmatic patients.

We report that in the presence of type 2 inflammation, IL-4/IL-13-induced 15LO1 binding to PEBP1 interferes with GRK2 binding, desensitizing the β_2 AR. The associated decrease in cAMP levels is likely to adversely affect mucociliary clearance, such that exogenous administration of β_2 -agonists in the presence of type 2 inflammation might have little epithelial cell effect and in fact could limit overall improvement in FEV₁ in response to treatment. Future *in vitro* and *in vivo* studies of ciliary beat are needed to fully address this. However, it is important to note that airway smooth muscle cells, likely the primary target for β_2 -agonists in asthmatic patients, do not express 15LO1. Thus any effects of IL-13 on β_2 AR/bronchodilator responses are unlikely to involve this 15LO1/GRK2 mechanism, explaining why we found no relationship between 15LO1 expression and

β_2 -agonist-induced bronchodilator responses *in vivo* (data not shown). Whether additional effects of β_2 AR desensitization could be seen in macrophages or eosinophils (each of which express 15LO1) costimulated with IL-13 and β_2 -agonists remains to be determined.

β_2 AR desensitization likely occurs through many mechanisms. Depending on the stimulation, desensitization can occur through receptor uncoupling, internalization, phosphorylation, and other closely related kinase pathways, including other GPCR kinases.^{12,45} Although further studies are needed to confirm whether GRK2/PEBP1 interactions desensitize β_2 ARs in HAECs, results presented here support the importance of this pathway. However, under type 2 conditions, interactions of 15LO1 with PEBP1 directly prevent GRK2 from being in an “inactive” state bound to PEBP1. This preferential binding of 15LO1 with PEBP1 not only leads to a freeing of Raf-1 and sustained activation of ERK but also allows free GRK2 to phosphorylate β_2 AR, leading to its desensitization. Intriguingly, a recent study further suggested that free GRK2 could act as a scaffolding protein for ERK, perhaps further augmenting its activation.⁴⁶ Thus in the face of IL-13-induced 15LO1, enhanced availability of GRK2 could not only lead to a desensitized β_2 AR but also increase the IL-13-induced activation of ERK, all of which could dramatically change epithelial cell function.

Limitations of the study include the small sample sizes and the confounding of β_2 -agonist use in the *ex vivo* studies. Additional data are needed to confirm that the IL-13-induced β_2 AR desensitization observed here has a functional effect on ciliary beat and mucociliary clearance both *in vitro* and *in vivo*. Finally, because of difficulties with specific antibodies, total GRK2 could not be used as the index for pGRK2 in most samples. However, the correlation between GRK2 and GAPDH was very strong, such that we do not believe the results would have been appreciably changed.

In summary, these results demonstrate a potentially clinically relevant effect of IL-13 on desensitization of β_2 AR in HAECs. The mechanism involves an enhanced availability of free GRK2 for phosphorylation because of its displacement from PEBP1 by high levels of IL-13-induced 15LO1. The downstream effect to desensitize β_2 AR has implications for epithelial function in patients with asthma and other airway diseases with evidence for type 2 inflammation, especially when combined with the known effect of 15LO1 to increase MUC5AC levels through a similar mechanism. Modification of interactions of 15LO1 with PEBP1 could be a novel target for restoring β_2 AR sensitization in patients with airway diseases.

We thank Dr Fernando Holguin (University of Pittsburgh) for kindly providing some of the primary epithelial cell samples for this study.

Key messages

- IL-13 stimulation desensitizes β_2 AR through 15LO1's competitive inhibition of GRK2 binding to PEBP1.
- These interactions, observed both *in vitro* and *in vivo*, could limit the beneficial effects of β_2 -agonists in the airway epithelium.

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METHODS

Reagents and antibodies

pGRK2 antibody (S670; mouse) was from Abcam (Cambridge, Mass). p β 2AR antibody (rabbit) was from Santa Cruz Biotechnology (Santa Cruz, Calif). Total β 2AR (rabbit) was from Cell Signaling (Danvers, Mass). Total GRK2 (rabbit) was from Pierce (Rockford, Ill). 15LO1 antibody was a gift from Doug Conrad, University of California, San Diego. GAPDH was from Novus Biologicals (Littleton, Colo), and IL-13 was from R&D Systems (Minneapolis, Minn). The cAMP assay was a kit from Cell Signaling (Cyclic AMP XP Assay Kit #4339). ALOX15 siRNA was purchased from Ambion (Austin, Tex), and siQUEST transfection reagent was from Mirus. ISO was from Sigma (Saint Louis, Mo). Protein A/GPlus-Agarose was from Santa Cruz Biotechnology. The Qproteome Cell Compartment Kit was from Qiagen (Valencia, Calif).

Study participants

Asthmatic participants met American Thoracic Society criteria for asthma and were recruited as part of the National Heart, Lung, and Blood Institute's Severe Asthma Research Program or the Electrophilic Fatty Acid Derivatives in Asthma studies.^{E1} Mild-to-moderate asthma was defined as participants with an FEV₁ of 60% of predicted value or greater receiving SABAs only or receiving low- to moderate-dose ICSs (<880 μ g/day fluticasone propionate or equivalent) plus SABAs. Severe asthma was defined by the American Thoracic Society 2000 definition, consisting of 1 or 2 major criteria (use of high-dose ICSs, frequent use of oral corticosteroids, or both) and at least 2 of 7 minor criteria.^{E2} HCs had no history of respiratory disease or recent respiratory tract infection and normal lung function. No subject smoked within the last year or smoked for greater than 5 pack years. The study was approved by the University of Pittsburgh's Institutional Review Board, and all participants provided informed consent before participation.

Participants underwent extensive characterization, including spirometry and bronchodilator reversibility testing, as previously described.^{E3}

Bronchoscopy with epithelial airway brushings

Bronchoscopy with epithelial brushing was performed, as previously described.^{E4,E5} Per protocol, all participants, including HCs, received 2.5 mg of nebulized albuterol within 15 to 45 minutes of the procedure. Airway brushings were obtained 30 to 60 minutes after albuterol from the fourth- to fifth-generation airways. The brushings typically contained more than 95% epithelial cells. A portion was placed into Western lysis buffer for protein evaluation on fresh cells. Another portion was placed into medium to be expanded for future cell culture work.

Primary bronchial epithelial cell culture in ALI cultures

Primary human bronchial airway epithelial cells were cultured in ALI, as previously described.^{E5-E7} From day 0 of ALI culture, cells were stimulated with IL-13 (10 ng/mL) for 48 hours. ISO (1 μ mol/L) was added to the medium for 10 minutes or for 30 minutes followed by a second addition 10 minutes later in the presence or absence of IL-13 stimulation (48 hours) for 15LO1 protein, as well as GRK2 and β 2AR signaling studies. Total protein samples were harvested for Western blotting. Protein fractions were isolated with the Qproteome Cell Compartment Kit, according to the manufacturer's instructions. The cells were lysed with 1 $\times$ cell lysis buffer before cAMP measurement by means of ELISA.

siRNA transfection of primary human epithelial cells in ALI cultures

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, cells were switched to the ALI state and stimulated with IL-13 (or not) for 48 hours and then ISO (or

media alone) for 10 minutes. Nonspecific scrambled siRNAs were transfected in control wells at the same time as the negative control.

Western blotting

Cell lysates were run on 4-12% SDS-PAGE gels under reducing conditions, as previously described.^{E5} Western blotting was performed with primary antibodies against pGRK2 (mouse anti-GRK2 [phospho S670] antibody), anti-p β 2AR (rabbit), total β 2AR (rabbit β 2AR antibody), and 15LO1 (rabbit). For the majority of subject samples (*in vitro* and *ex vivo*), a reliable total GRK2 antibody was not available. Although the total GRK2 antibody (Pierce) eventually produced reliable results, the amounts of samples remaining for most experiments were not sufficient to run total GRK2. Correlations of total GRK2 with GAPDH ($n = 9$) were very strong ($r = 0.93$, $P < .0001$, see Fig E4). Therefore pGRK2 was indexed to GAPDH for both *in vitro* and *ex vivo* samples. The membrane was developed, and densitometry was performed with Multi Gauge software on a Fuji analyzer (Fuji Digital Co, Stamford, Conn).

Coimmunoprecipitation

Cells were washed with cold PBS before lysing in mild protein lysis buffer (50 mmol/L Tris/HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, and 0.1% Nonidet P-40) with protease and phosphatase inhibitors. The cell lysate was precleaned with protein A agarose beads (Protein A/GPlus-Agarose, Santa Cruz Biotechnology) and subsequently incubated with primary antibody (pull-down) overnight. Protein A agarose beads were added and incubated for 1 hour at 4°C. Immunoprecipitates were washed and boiled in 2 $\times$ SDS sample buffer for 5 minutes and centrifuged, and cell lysates were separated on 4-12% SDS-PAGE gels for Western blotting by using primary antibodies generated from different species from the pull-down primary antibody. Nonimmunized IgG was applied as the pull-down control to confirm binding specificity. Total protein without immunoprecipitates was run at the same time as the control. The membrane was developed, and densitometry was performed with Multi Gauge Software.

cAMP assay

Cells pretreated (or not) with IL-13 (10 ng/mL) for 48 hours were stimulated with 1 μ mol/L ISO for 30 minutes or media alone. Cells were washed with cold PBS and stimulated for an additional 10 minutes with 1 μ mol/L ISO. Subsequently, the cells were placed on ice and rinsed twice with cold PBS and 1 $\times$ lysis buffer with protease and phosphatase inhibitors were added. Cell lysates were analyzed to evaluate cAMP levels by using a competitive ELISA kit from Cell Signaling. All cAMP measurements were run as technical duplicates. The data presented are means of the 2 technical duplicates. When additional cells were available and 2 sample wells were collected, the reported cAMP level was the mean of the duplicates from the 2 sample wells. Data are presented as the mean of duplicates (coefficient of variation = 8.6% [6.5% to 10.7%]).

IF and confocal microscopy of human airway tissue

Endobronchial biopsy specimens from 1 patient with mild and 6 patients with severe asthma and 1 autopsy lung tissue specimen from a patient with severe asthma who died of an asthma exacerbation were evaluated and compared with endobronchial biopsy specimens from 5 healthy controls and 3 autopsy tissues from healthy persons (see Table E2 for details). Tissues were fixed in acetone and embedded in glycol methacrylate resin for IF staining. Sections were blocked, incubated with the primary antibodies of interest, washed again, and incubated with cocktails of Alexa Fluor 555 and 488 secondary antibodies. DRAQ5 nuclear stain and no-primary antibody controls were done at the same time. Sections were washed and sealed for observation under the Olympus FluoView 1000 confocal microscope (Olympus, Center Valley, Pa) and analyzed with MetaMorph software (Molecular Devices, Sunnyvale, Calif). For confocal/IF of ALI culture, cells were washed, permeabilized, and rehydrated. After blocking, cells were incubated with primary antibodies for IF staining.

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- E3. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005; 171:912-30.
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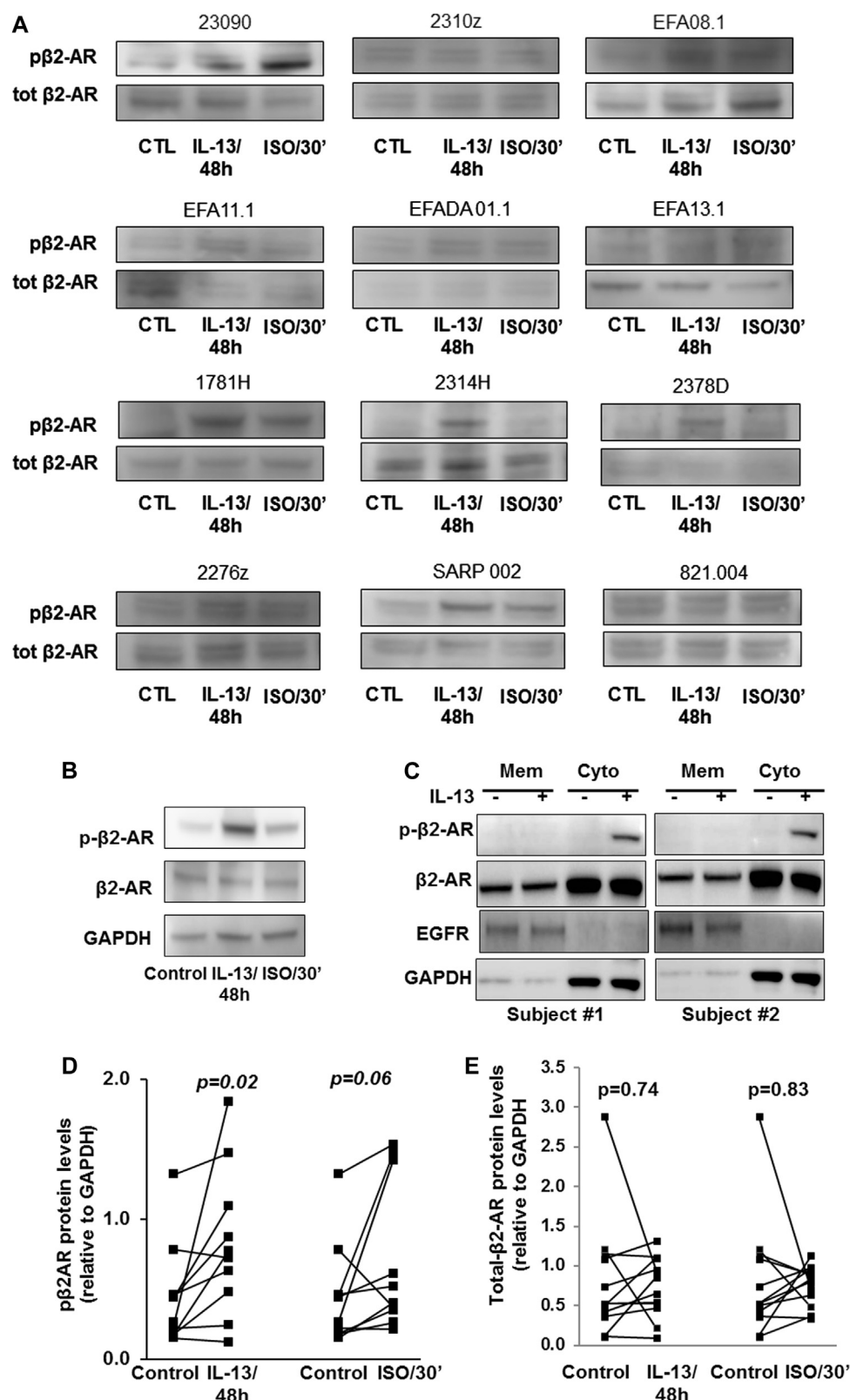


FIG E1. A, IL-13 and ISO induce β 2AR phosphorylation in primary bronchial airway epithelial cells, as shown in these Western blots. ALI-cultured bronchial airway epithelial cells were stimulated with ISO (30 minutes) or IL-13 (48 hours). Total protein was harvested for β 2AR phosphorylation detection by means of Western blotting, with GAPDH as the loading control (CTL). **B,** Representative Western blots of phosphorylated and total β 2AR. **C,** Representative Western blots of cell fractions using GAPDH and epidermal growth factor receptor as loading controls. *Cyto*, Cytoplasmic; *Mem*, membranous. **D,** Both IL-13 and ISO significantly increased β 2AR phosphorylation compared with medium alone. **E,** No effect of IL-13 or ISO on total β 2AR protein expression.

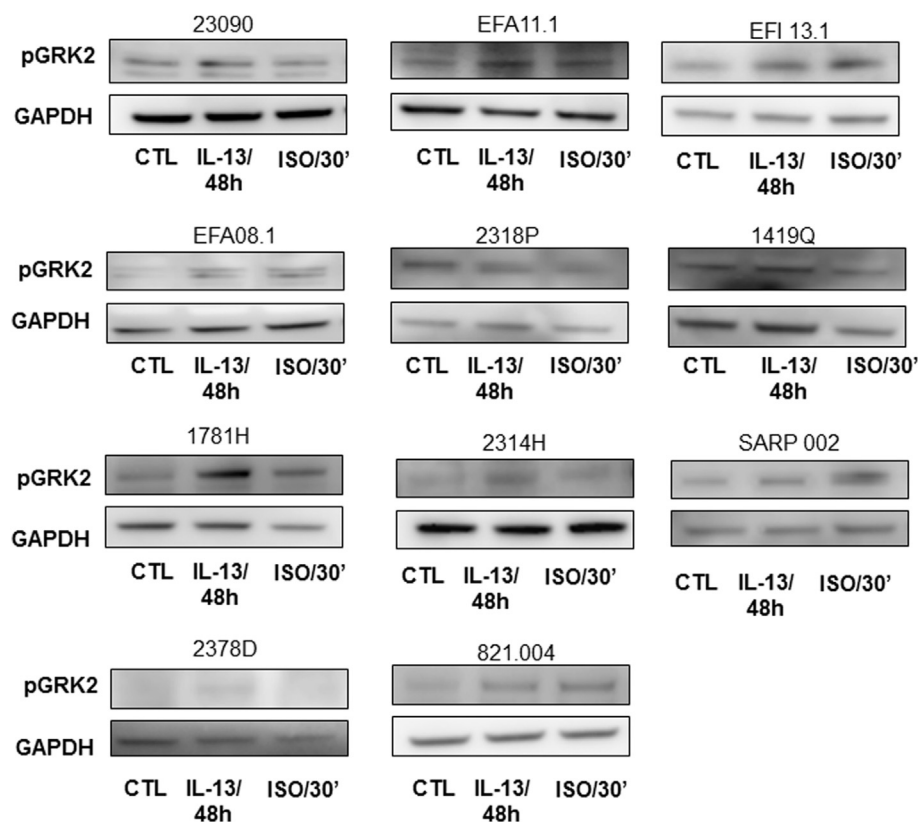


FIG E2. IL-13 and ISO induce GRK2 phosphorylation in primary bronchial airway epithelial cells. All-cultured bronchial airway epithelial cells were stimulated with ISO (30 minutes) or IL-13 (48 hours). Total protein was harvested for GRK2 phosphorylation detection by using Western blotting, with GAPDH as the loading control.

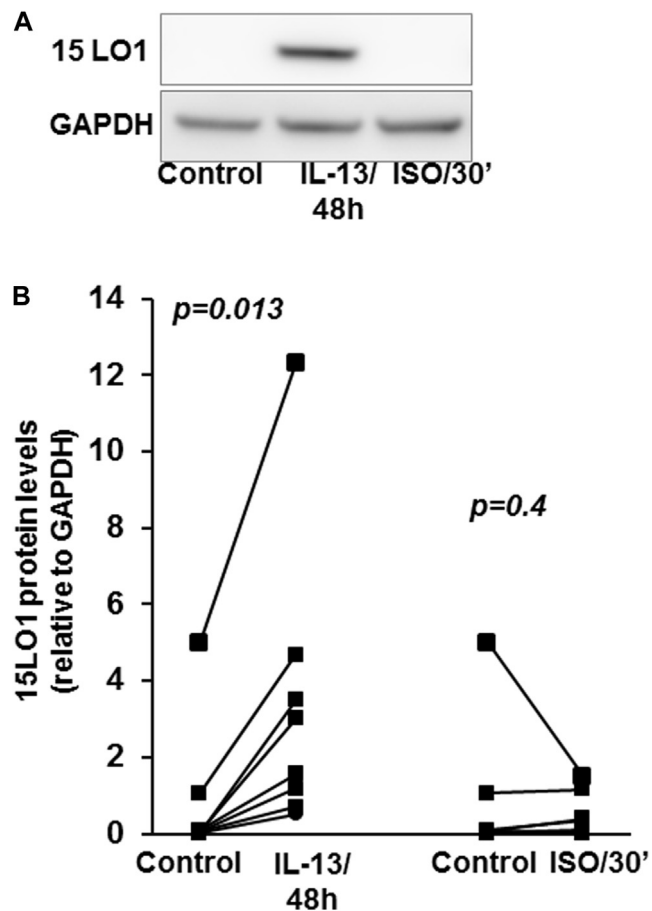


FIG E3. IL-13 induced 15LO1 protein, which was not affected by ISO stimulation. ALI-cultured cells were stimulated with or without IL-13 for 48 hours or ISO for 30 minutes. **A**, Total protein was harvested for Western blotting. **B**, IL-13 induced 15LO1 protein expression ($P = .013$), whereas ISO (30 minutes) did not ($P = .4$).

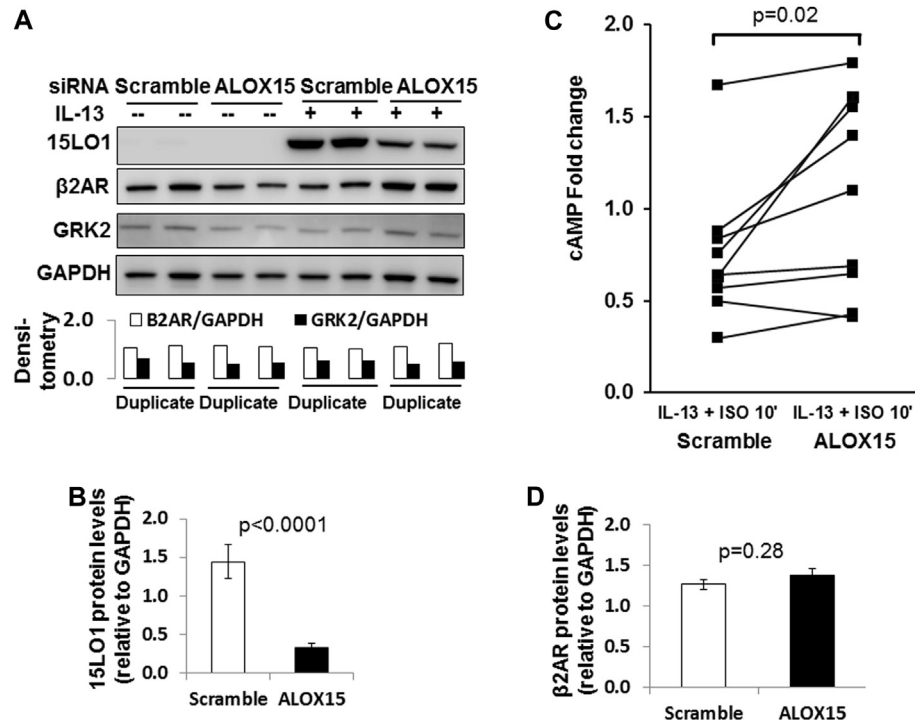


FIG E4. ALOX15 siRNA knockdown of 15LO1 expression restored ISO-induced cAMP generation in HAECS. ALI-cultured cells transfected with ALOX15 siRNA or scrambled siRNA were stimulated with IL-13 for 48 hours plus ISO for 10 minutes. Total protein was harvested for Western blotting and ELISA. Data are presented as means of duplicates. **A** and **B**, ALOX15 siRNA knocked down 15LO1 protein induced by IL-13/ISO stimulation (Western blotting and densitometry). **C**, ALOX15 siRNA knockdown restored ISO (10 minutes)-induced cAMP generation in cells treated with IL-13 for 48 hours compared with scrambled siRNA. **D**, No changes in total β2AR expression after ALOX15 siRNA transfection.

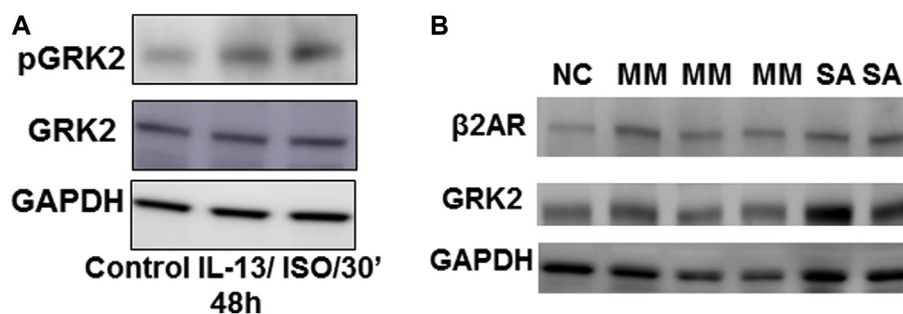


FIG E5. No change in total GRK2 and β 2AR protein expression in response to IL-13 and ISO stimulation *in vitro* and across subjects *ex vivo*. **A**, ALI-cultured bronchial airway epithelial cells were stimulated with ISO (30 minutes) or IL-13 (48 hours). Total protein was harvested and phosphorylated, and total GRK2 was measured using Western blotting, with GAPDH as the loading control. **B**, Total protein from freshly brushed airway epithelial cells was harvested, and total β 2AR and GRK2 protein expression was measured using Western blotting with GAPDH as the loading control.

TABLE E1. Demographics for patients with *in vitro* samples (n = 49)

	HCs	Patients with mild-to-moderate asthma	Patients with severe asthma	<i>P</i> value
Age (y), mean $\pm$ SD	31.1 $\pm$ 6.8	30.1 $\pm$ 9.8	44.6 $\pm$ 10.5	.0002
Sex (% female)	14	16.7	40.5	.4
Race (% [W/AA/other])	80/10/10	64/27/9	57/14/29	.4
FEV ₁ (% predicted), mean $\pm$ SEM	94.3 $\pm$ 5.5	92.7 $\pm$ 5.4	55.2 $\pm$ 3.8	.0001
Bronchodilator response (%), mean $\pm$ SEM	8 $\pm$ 4	7 $\pm$ 4	18 $\pm$ 3	.02
FENO (ppb), mean $\pm$ SEM	20.6 $\pm$ 6	56.8 $\pm$ 14	66.1 $\pm$ 17	.03
ICS (% [no/yes])	100/0	70/30	0/100	.0001

AA, African American; *FENO*, fraction of exhaled nitric oxide; W, white.

TABLE E2. Demographic characteristics of IF/confocal tissue samples

Subject type	Age (y), mean $\pm$ SD	Race (% [W/AA/other])	Sex (% female)	ICS (% [no/yes])
Biopsy tissue				
HCs (n = 5)	43 $\pm$ 5	100/0/0	40/60	100/0
Patients with mild-to-moderate asthma (n = 1)	25	100/0/0	100	0/100
Patients with severe asthma (n = 6)	49 $\pm$ 4	100/0/0	67/33	0/100
Autopsy tissue				
Patients with severe asthma (n = 1)	42	White	Female	Yes/Yes
HCs (n = 3)	57 $\pm$ 6	(0/1/2 unknown)	33/67	NA

AA, African American; W, white.