Prostanoid Receptors of the EP₄-Subtype Mediate Gene Expression Changes in Human Airway Epithelial Cells with Potential Anti-Inflammatory Activity

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ABSTRACT

There is a clear, unmet clinical need to identify new drugs to treat individuals with asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF) in whom current medications are either inactive or suboptimal. In preclinical models, EP₄-receptor agonists display efficacy, but their mechanism of action is unclear. In this study, using human bronchial epithelial cells as a therapeutically relevant drug target, we hypothesized that changes in gene expression may play an important role. Several prostanoid receptor mRNAs were detected in BEAS-2B cells, human primary bronchial epithelial cells (HBECS) grown in submersion culture and HBECS grown at an air-liquid interface with PTGER4 predominating. By using the activation of a cAMP response element reporter in BEAS-2B cells as a surrogate of gene expression, Schild analysis determined that PTGER4 mRNAs encoded functional EP₄-receptors. Moreover, inhibitors of phosphodiesterase 4 (roflumilast N-oxide [RNO]) and cAMP-dependent protein kinase augmented and attenuated, respectively, reporter activation induced by 2-[3-

SIGNIFICANCE STATEMENT

Using human bronchial epithelial cells as a therapeutically relevant drug target, we report that EP₄-receptor activation promoted gene expression changes that could provide therapeutic benefit in individuals with asthma, COPD, and IPF in whom current treatment options are ineffective or suboptimal.

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory disorders that, together, afflict approximately 1 billion people worldwide. Despite dissimilarity in etiology, mainstay therapies are similar and include inhaled corticosteroids (ICSs) and long-acting, β₂-adrenoceptor agonists (LABAs). Although these are often effective in mild-to-moderate disease, there are individuals with asthma and COPD in whom pulmonary inflammation is heightened, especially during exacerbations (Hurst et al., 2006; Perera et al., 2007), who are insensitive or respond suboptimally to these drugs. Similarly, idiopathic pulmonary fibrosis (IPF) is a devastating, interstitial lung disease with a dismal prognosis, increasing global prevalence (Hutchinson et al., 2015), and limited therapeutic options (Hewitt and Maher, 2019). Thus, there is an urgent, unmet, clinical need for interventions that can control these difficult-to-treat disorders.

One candidate that may mitigate certain pathologic characteristics common to these diseases, including inflammation and compromised lung function, is prostaglandin (PG) E₂. In individuals with asthma and bronchitis, PGE₂ is a bronchodilator and displays anti-inflammatory activity (Peebles, 2019). However, PGE₂ has a short half-life (Hamberg and Samuelsson, 1971), promotes cough (Costello et al., 1985), and, with chronic dosing, may cause cancer (Wang and Dubois, 2006), which calls into question its therapeutic utility. Nevertheless, the appreciation that
PGE2 activates four distinct G protein–coupled receptors (GPCRs) (i.e., EP1–EP3) has prompted studies to determine if the beneficial and airway irritant/tumorigenic activities can be dissociated. PGE2 relaxes human airway smooth muscle (ASM) by an EP receptor–mediated mechanism (Buckley et al., 2011) and suppresses proinflammatory and immune cell function by engaging the EP2- and EP4-subtypes (Peebles, 2019). In murine models of respiratory diseases, the EP4-receptor also attenuates indices of inflammation (Birrell et al., 2015; Felton et al., 2018; Zhou et al., 2018), whereas the EP3-subtype mediates cough (Maher et al., 2009). Similarly, EP2 and EP4-receptor agonists inhibit the differentiation of human lung fibroblasts to myofibroblasts driven by transforming growth factor-β1 (Sieber et al., 2018). Myofibroblasts have a significant capacity to deposit extracellular matrix, and this likely contributes to the parenchymal stiffening and progressive loss of lung function in IPF. Moreover, PGE2 and its 16,16-dimethyl analog decrease inflammation, fibrotic lung injury, and mortality (Failla et al., 2009; Ivanova et al., 2013).

In terms of signaling, EP receptor mediates their beneficial effects by increasing cAMP, whereas nocanical processes apparently lead to tumorigenesis with extracellular signal-regulated kinases playing a central role (Wang and Dubois, 2010). These data suggest that the therapeutic activity of PGE2 may be mimicked with subtype-selective agonists that preferentially couple to Gs or whose ability to increase cAMP is enhanced by a phosphodiesterase (PDE) inhibitor, which augments canonical signaling bias.

How EP2- and EP4-receptor agonists arrest pulmonary inflammation is unclear, but studies with β2-agonist receptor agonists indicate that genomic mechanisms may be involved (Yan et al., 2018). Indeed, the EP2- and EP4-receptors couple to the same, primary, signal transduction pathway as the β2-receptor, which culminates in the activation of cAMP-dependent protein kinase (PKA) (Neuhaus-Rube et al., 1997; Sugimoto et al., 2003). Gene transcription can then proceed after the phosphorylation of several transcription factors, including cAMP response element (CRE)–binding protein.

Human bronchial epithelial cells (HBECs) play an important, first-line role in host defense, innate immunity, and immune system regulation. However, these cells also respond to noxious stimuli by generating a plethora of cytokines, chemokines, growth factors, lipids, peptides, and reactive oxygen and nitrogen species that promote, perpetuate, and exacerbate airway inflammation (Proud and Leigh, 2011). It is this prominent, proinflammatory role that makes HBECs a primary target for inhaled drugs used to treat chronic lung diseases.

EP4-receptors on airway structural cells, such as the epithelium, could mediate many of the beneficial effects of PGE2 in preclinical disease models by modulating gene expression. To explore this prospect, prostanooid receptor mRNAs were first measured in three epithelial cell variants: BEAS-2B cells, HBECs grown in submersion culture, and HBECs grown at an air-liquid interface (ALI). Gs-coupled prostanooid receptors were then characterized pharmacologically by employing a CRE luciferase reporter in BEAS-2B cells as a surrogate of gene transcription. Finally, the genomic impact of an EP4-receptor agonist [2-[(1R,2S,3R)-3-hydroxy-2-[(E,3S)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxocyclopyrenyl]sulphonylpropylsulphonyl] acetic acid (ONO-AE1-329) was assessed in each epithelial cell variant. To provide clinical insight, these determinations were made in the presence of a glucocorticoid (dexamethasone) and a PDE4 inhibitor (roflumilast N-oxide [RNO]), which exert profound genomic effects in subjects with respiratory diseases (Yick et al., 2013; Govoni et al., 2020) and often cooperate in an additive or synergistic manner with Gs-coupled receptor agonists (Wilson et al., 2009; Greer et al., 2013; Joshi et al., 2019). The LABAs indacaterol, vilanterol, and formoterol were used throughout as clinically relevant comparators.

### Materials and Methods

#### Stable Generation of BEAS-2B Luciferase Reporter Cells

BEAS-2B cells (American Type Culture Collection, Manassas, VA) were transfected with plasmid DNA (either pAdneo2-C6-BGL or pGL3.neo.TATA2GRE) to generate 6× CRE and 2× glucocorticoid response element (GRE) luciferase reporter cells, respectively, as described previously (Chivers et al., 2004; Meja et al., 2004; Moodley et al., 2013).

#### Submersion Culture of BEAS-2B Cells

Native and BEAS-2B reporter cells were cultured for 2 days under a 5% CO2/air atmosphere at 37°C in 24-well plastic plates (Costar Inc., Corning, NY) containing Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% FBS (Life Technologies, Burlington, ON, Canada), 1-glutamine (2.5 mM), and NaHCO3 (14 mM; all Invitrogen) and for a further 24 hours in serum-free medium without supplements (Greer et al., 2013). At this time, cultures were confluent and used to measure baseline GPCR mRNA abundance and ONO-AE1-329–, RNO–, and dexamethasone-induced CRE reporter activation and gene expression changes, with indacaterol and vilanterol acting as comparators.

#### Measurement of Luciferase Activity

Luciferase activity was measured by luminometry and expressed as a fold change relative to time-matched, vehicle-treated cells (Yan et al., 2018).

#### Expression of a Protein Inhibitor of PKA in BEAS-2B Cells

Subconfluent, 6× CRE BEAS-2B reporter cells were infected [multiplicity of infection (MOI) = 25; 48 hours] with an adenovirus vector (Ad5.CMV.PKia) containing a DNA fragment encoding the amino acid sequence of the cAMP-dependent protein kinase inhibitor α (PKiα) (Meja et al., 2004). The biologic effects of the virus, per se, were controlled with a vector (Ad5.CMV.GFP) encoding green

### Abbreviations

- ALI: air-liquid interface
- ASM: airway smooth muscle
- COPD: chronic obstructive pulmonary disease
- CRE: cAMP response element
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- GPCR: G protein–coupled receptor
- GRE: glucocorticoid response element
- HBEC: human bronchial epithelial cell
- ICS: inhaled corticosteroid
- IPF: idiopathic pulmonary fibrosis
- L-161,982, (2R)-3,3-dimethyl-7-oxo-hept-5-enoic acid; L-902,688, human lung fibroblasts to myofibroblasts driven by trans-
fluorescent protein. More than 90% of BEAS-2B cells infected with Ad5.CMV.PKα express the transgene (Meja et al., 2004). HBECs Grown in Submersion Culture and at an ALI. Ethics approval for the use of human tissues has been granted by the Conjoint Health Research Ethics Board of the University of Calgary. Epithelial cells were prepared by proteinase digestion of nontransplanted, normal airways harvested from 11 nonasthmatic donors obtained from a tissue retrieval service at the International Institute for the Advancement of Medicine (Edison, NJ; see Supplemental Table 1 for demographics).

HBECs grown in submersion culture were seeded in 12-well plates (Costar) containing bronchial epithelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with penicillin (50 µg/ml) and streptomycin (10 µg/ml), and maintained for ∼14 days under 5% CO2/air atmosphere at 37°C until ∼80% confluent. Cells were cultured for a further 24 hours in supplement-free, basal medium (PromoCell) and used to measure either baseline GPCR mRNA abundance or changes in gene expression in response to ONO-AE1-329, RNO, and dexamethasone with vilanterol acting as a comparator.

For all IL cultures, HBECs, in T75 cm2 flasks (Costar), were maintained for 72 hours at 37°C under 5% CO2/air atmosphere in PneumaCult-EX complete medium (Stemcell Technologies, Vancouver, BC, Canada). This was replaced every 48 hours until cells were 90% confluent, at which time they were lifted (TrypLE Select; Invitrogen) and washed in F12 medium containing 20% FBS. Cells were resuspended in PneumaCult-EX complete medium and seeded at a density of 2 × 104/cm2 in 0.4 µm pore transwell inserts (Costar) coated with bovine collagen type I/III (Advanced BioMatrix, San Diego, CA). At 48 hours the medium was replaced with PneumaCult-ALI differentiation medium (Stemcell Technologies) containing 10 × supplement, fluconazole (25 µg/ml), and penicillin and streptomycin (each 10 µg/ml). Cultures were fed every 48 hours for 5 weeks with PneumaCult-ALI differentiation medium containing 100 × supplement, hydrocortisone (0.5 µg/ml), and heparin (50 µg/ml). Cells were maintained for an additional 18 hours in PneumaCult basal medium (Stemcell Technologies), which lacks all supplements including hydrocortisone, and used to measure either baseline GPCR mRNA abundance or changes in gene expression in response to ONO-AE1-329 and dexamethasone, with formoterol acting as a comparator.

RNA Extraction. Total RNA was extracted with Nucleospin RNA mini kits (Macherey-Nagel Inc., Bethlehem, PA) and, when appropriate, reverse transcribed to cDNA using a qscript cDNA synthesis kit as described by the manufacturer (Quanta Biosciences, Gaithersburg, MD). mRNA abundance was then determined as described below.

Determination of GPCR mRNA Expression by TaqMan Polymerase Chain Reaction. GPCR mRNAs in BEAS-2B cells were measured by using a TaqMan GPCR array (Applied Biosystems) as described previously (Joshi et al., 2017; Yan et al., 2018), and data were expressed either as a ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or as a fold change relative to time-matched, vehicle-treated cells.

Curve Fitting. Monophasic and biphasic concentration-response (E[A]) curves were fit by least-squares, nonlinear, iterative regression to eq. 1 and eq. 2, respectively (Prism 6; GraphPad Software Inc., San Diego, CA):

\[
E = E_{\text{min}} \cdot \frac{E_{\text{max}} - E_{\text{min}}}{1 + 10^{\log([A]\cdot I)/(50) - \log([A_{50}]\cdot I)}}
\]

(1)

\[
E = E_{\text{min}} \cdot \left( \frac{E_{\text{max}} - E_{\text{min}}}{1 + 10^{\log([A]\cdot I)/(50) - \log([A_{50}]\cdot I)}} \right) - E_{\text{max}} + E_{\text{min}} \cdot \frac{1}{1 + 10^{\log([A]\cdot I)/(50) - \log([A_{50}]\cdot I)}}
\]

(2)

where E is the effect, E_{\text{min}} and E_{\text{max}} are the basal and maximum responses, respectively, [A] is the molar concentration of agonist, [A_{50}] is the molar concentrations of agonist that produces (E_{\text{max}} - E_{\text{min}})/2, and n is the gradient of the E/[A] curve at the corresponding [A_{50}] level. For biphasic E/[A] curves, the subscripts 1 and 2 refer to the high and low potency components, respectively, and F is the fraction of the total response attributable to the first component. Agonist potency is expressed as p[A_{50}] (−log_{10} of [A_{50}]).

Determination of Receptor Reserve. Occupancy-response curves were constructed to ONO-AE1-329 in the absence and presence of RNO (1 µM), assuming an affinity (K_{B}) for the human EP_{2} receptor of 9.7 nM (Suzawa et al., 2000). At each concentration of ONO-AE1-329, fractional receptor occupancy was determined according to eq. 3 where R_{A} and R_{T} represent the number of agonist-occupied receptors and total number of receptors, respectively.

\[
R_{A}/R_{T} = [A]/(K_{B} + [A])
\]

(3)

Determination of Antagonist Equilibrium Dissociation Constants. The affinities of prostanoid receptor antagonists were determined by least-squares, iterative, nonlinear regression using a modification of the Gaddum and Schild equations (Waud et al., 1978). Agonist E/[A] curves were generated in BEAS-2B reporter cells pretreated (30 minutes) with vehicle or the antagonist of interest. Each family of E/[A] curves was fit simultaneously to eq. 4, where [B] is the molar concentration of antagonist, S is the gradient of the Schild slope, and 1/A_{50} is the affinity of the antagonist when S = 1, which is equivalent to 1/K_{B}.

\[
E = E_{\text{min}} + \left( \frac{E_{\text{max}} - E_{\text{min}}}{1 + 10^{\log([B]/A_{50}) - \log([B])}} \right)
\]

(4)

Drugs and Reagents. 4-[2-[(1R,2R,3E)-3-Hydroxy-2-[E,3S,5S]-3-hydroxy-5-methyl-non-1-enyl]-5-oxo-cyclopentyl]acetyl]cyclohexane
carboxylic acid (ONO-DI-004), (Z)-7-[[1R,2R,3R,5R]-5-chloro-3-hydroxy-2-([E,4S]-4-hydroxy-4-[1-prop-2-enylcyclobutyl]but-1-enyl] cyclopentyl] hept-5-enoic acid (ONO-AE1-259), (Z)-7-[[1R,2S,3R]-3-methoxy-2-([E,3S]-3-methoxycyclopenta-1-enyl]-5-oxo-cyclopentyl] hept-5-enoic acid (ONO-AE-248), and ONO-AE1-329 were from ONO Pharmaceuticals (Osaka, Japan), and N-[2-[[3-[butyl-5-oxo-1-[2-(trifluoromethyl)phenyl]-1,2,4-triazol-4-yl)methyl]phenyl]phenylsulphonyl-5-methyl-thiophene-2-carboxamide (L-161,992) and (Z)-5-[[E,3R]-4-difluoro-3-hydroxy-4-phenyl-but-1-enyl]-1-(6H-tetrazol-5-y1)hexylpyrrolidin-2-one (L-902,688) were donated by Merck Frosst, Inc. (Montreal, PQ, Canada). (2E)-3-[[3,4,5-trimethoxyphenyl]-N-[2-(2-methyl-1H-indol-1-yl)ethyl]prop-2-enamide (TG4-155), (2R)-3-[[4-fluoro-phenyl]-2-[[5-[4-fluorophenyl]-benzofuran-2-yl]methoxy] carbonylamino] propanoic acid (Ro3244794), RNO, indacaterol maleate, and vilanterol trifenate were from ChemDiv (San Diego, CA), Roche Pharmaceuticals (Seattle, WA), and Toronto Research Chemicals (North York, ON, Canada), respectively. Formoterol fumarate, rolipram, dexamethasone, prostanoids, and all other reagents were from Sigma-Aldrich (Oakville, ON, Canada). Drugs were dissolved in DMSO and diluted in culture medium. The highest concentration of DMSO (0.2% v/v) used in these experiments did not affect any output measured.

**Results**

**GPCR mRNA Expression Among Human Airway Epithelial Cell Variants.** Mining data from prior, microarray-based, gene expression profiling studies identified 134 and 97 GPCRs in BEAS-2B cells (Yan et al., 2018) and HBECs grown in submersion culture (Proud et al., 2012), respectively, using the International Union of Pharmacology/ British Journal of Pharmacology (IUPHAR/BPS) Concise Guide to Pharmacology database (www.guidetopharmacology.org). However, the presence of many of these transcripts was equivocal, due to variability in signal intensities among probes. Therefore, upon positive results, Affymetrix Microarray Suite 5.0 software made positive calls, with one or more probe sets, for PTGER4, PTGFR, and TBX2AR in BEAS-2B cells, and for PTGER4 in HBECs; transcripts encoding other subtypes were either absent or their expression was marginal (Supplementary Tables 3 and 4).

Given the difficulty in assigning a “marginal” call as either absent or present, prostanoid receptor mRNAs were also determined by RNA-seq. At a cutoff threshold of ≥0.25 TPM, this method identified 112, 106, and 159 GPCR mRNAs in untreated BEAS-2B cells, HBECs grown in submersion culture, and HBECs grown at an ALI, respectively. The Venn diagram in Fig. 1, together with the scatter plot matrix and three-dimensional plot, which summarize the pairwise and three-way interdependence between each variable, respectively (B.M. Taylor, preprint, arXiv:2003.02561), show that 74 genes were common to the three epithelial cell types (41% concordance) and that their relative abundance was highly correlated (mcor(x,y,z) = 0.54). A further 78, 92, and 99 transcripts were common to BEAS-2B and HBEC, BEAS-2B and ALI cultures, and BEBEC and ALI cultures, respectively, with 16 (BEAS-2B), 3 (HBEC), and 42 (ALI cultures) mRNAs being uniquely expressed (Fig. 1, A and B). The expression of the 74 shared mRNAs is shown as a heat map in Fig. 2A, where TPMs in ALI cultures are ranked highest to lowest against which the BEAS-2B cell and HBEC data are aligned.

**Prostanoid Receptor mRNAs in BEAS-2B Cells.** RNA-seq confirmed the presence of PTGER4, PTGFR, and TBX2AR mRNAs in BEAS-2B cells and also identified transcripts for PTGER2 and PTGIR (Fig. 2B). PTGDR, PTGDR2, PTGER1, and PTGER3 mRNAs were not expressed at ≥0.25 TPM. These findings were corroborated by using a human TaqMan GPCR array, which also detected a relatively weak signal for PTGER3 (Supplemental Fig. 1). Thus, the relative abundance of prostanoid receptor mRNAs in BEAS-2B cells determined by RNA-seq was from highest to lowest in TPM: PTGER4 > TBXAR2 > PTGFR > PTGER2 > PTGIR. The gene encoding the β2-adrenoceptor, ADRB2, was also expressed and included as a comparator. Of the 112 GPCR mRNAs identified in BEAS-2B cells, PTGER4 and ADRB2 were ranked 64th (1.4 TPM) and 19th (10.6 TPM), respectively (Fig. 2B).

**Prostanoid Receptor mRNAs in HBECs Grown in Submersion Culture and at an ALI.** RNA-seq confirmed the presence of PTGER4 in HBECs grown in submersion culture and, in addition, identified transcripts at lower relative abundance for TBX2AR (Fig. 2B). ADRB2 mRNA was also expressed and, as before, included as a comparator. Of the 106 GPCR mRNAs identified in HBECs grown in submersion culture, PTGER4 and ADRB2 were ranked 35th (10.4 TPM) and 6th (57.3 TPM), respectively (Fig. 2B).

RNA-seq detected a greater number of transcripts encoding prostanoid and other GPCRs in HBECs grown at an ALI that met the ≥0.25 TPM cutoff threshold than in their submersion culture counterparts (Fig. 2B). PTGFR was the most abundant followed by PTGER4, PTGER2, ADRB2, TBX2AR, and PTGER1 (Fig. 2B). Of the 159 GPCR mRNAs identified in ALI cultures, PTGER4 and ADRB2 were ranked 31st (9.4 TPM) and 75th (1.9 TPM), respectively (Fig. 2B). Thus, the abundance of PTGER4 relative to ADRB2 was 4.95-fold higher in well differentiated HBECs yet 5.51-fold lower in the same cells grown as a monolayer (Fig. 2B).

**Identification of Prostanoid Receptors in 6x CRE BEAS-2B Reporter Cells.** The effect of prostanoid receptor agonists on luciferase activity in 6x CRE BEAS-2B reporter cells is shown in Fig. 3A. For reference, the affinities and selectivities of all synthetic ligands used in this study are presented in Supplemental Table 5. PGE2 activated the reporter in a concentration-dependent and biphasic manner.
Fig. 1. Correlation of GPCR mRNA expression among three airway epithelial cell variants. (A) Proportional, three-way Venn diagram showing GPCR mRNAs that are common to, and unique between, BEAS-2B cells, HBECs grown in submersion culture, and HBECs grown at an ALI. Of the 182 transcripts identified across the three variants, 41% (74 genes) were shared. (B) Scatter plot matrix summarizing the pairwise interdependence in baseline expression of the 74 common GPCR mRNAs plotted on a log2 scale as mean TPM. The *r* values are Pearson product moment correlation coefficients. (C) A three-dimensional scatter plot showing multiway correlation of the same data presented in (B) between the three airway epithelial cell variants. A simulated three-dimensional scatter plot showing a perfect correlation (mcor{\(x, y, z\)} = 1) is provided as a comparator. Vertical lines depict the location of the points on the \(x-y\) plane where the heights correspond to the location on the \(z\) axis. Circles colored red indicate mRNAs encoding PTGER4, TBXA2R, and ADRB2. (D) Proportional, two-way Venn diagrams showing GPCR mRNAs that are common to, and unique between, each pair of epithelial cell variants; the number and percentage of total transcripts shared between each pair is shown above each diagram. Data are the means of \(N\) independent determinations. *\(p < 0.05\), variables significantly correlated.
with an [A]_{50} for the high potency component of 36 nM (Table 1). An IP-receptor agonist, taprostene (Schneider et al., 1993), likewise increased luciferase activity but was less effective and less potent ([A]_{50} \sim 80 nM) than PGE_{2}, PGD_{2}, PGF_{2\alpha}, and U-46619 were weak agonists and only active at concentrations (>1 \mu M) where selectivity for their respective cognate receptor is lost (Fig. 3A; Table 1).

The selective EP_{2}-receptor agonists, ONO-AE1-329 (Suzawa et al., 2000) and L-902,688 (Young et al., 2004), activated 6× CRE BEAS-2B reporter cells in a concentration-dependent manner (Fig. 3B). Both ligands had comparable potency ([A]_{50} \sim 7 nM) and were equi-effective (Fig. 3B; Table 1). However, relative to indacaterol, a LABA used to define the system maximum response (E_{\text{m}}) (Yan et al., 2018), ONO-AE1-329 was a partial agonist with an intrinsic activity of 0.62 (Fig. 3C). The selective EP_{2}-receptor agonist, ONO-AE1-259 (Suzawa et al., 2000) also activated 6× CRE BEAS-2B reporter cells, but was less potent ([A]_{50} \sim 30 nM) and less effective than ONO-AE1-329 with an intrinsic activity of 0.23 (vs. indacaterol) (Fig. 3C; Table 1). When combined, ONO-AE1-329 (1 \mu M) and ONO-AE1-259 (1 \mu M)
increased CRE reporter activity to a level similar to the sum of the two drugs alone. Moreover, the magnitude of this effect was comparable to the responses induced by indacaterol (100 nM) and a structurally unrelated LABA, vilanterol (100 nM), which were not augmented by ONO-AE1-259 and/or ONO-AE1-329 (Fig. 3D). Agonists with selectivity for the EP1 and EP3 receptor subtypes (ONO-DI-004 and ONO-AE-248, respectively; Suzawa et al., 2000) were inactive (Fig. 3B).

**Effect of EP2- and EP4-Receptor Antagonists on PGE2-Induced, CRE Reporter Activation.** Pretreatment (30 minutes) of 6 × CRE BEAS-2B reporter cells with a selective, EP4-receptor antagonist, L-161,982 (50 nM) (Machwate et al., 2001), had no effect on luciferase activity per se but produced a dextral displacement of the PGE2 E/[A] curve without changing Emax (Fig. 4A). Moreover, the mean PGE2 E/[A] curve, which was biphasic in the absence of antagonist, was rendered monophasic in cells pretreated with L-161,982 (Fig. 4A).

Pretreatment (30 minutes) of 6 × CRE BEAS-2B reporter cells with a selective EP2-receptor antagonist, TG4-155, at a concentration (30 nM) ~10× higher than its affinity for the EP2-receptor (Dian et al., 2012) did not affect baseline luciferase activity. Nevertheless, TG4-155 antagonized the activity of high concentrations (from 30 nM to 10 μM) of PGE2 without changing the biphasic shape of the E/[A] curve (Fig. 4A). In combination, L-161,982 (50 nM) and TG4-155 (30 nM) antagonized PGE2-induced reporter activation to a greater extent than either drug alone (Fig. 4A).

**Effect of EP2- and EP4-Receptor Antagonists on ONO-AE1-259-Induced, CRE Reporter Activation.** ONO-AE1-259 was a weak activator of the 6 × CRE reporter in BEAS-2B cells (Fig. 3, B and C). Hence, the PDE4 inhibitor, RNO (1 μM), was present throughout to enhance the activity of the luciferase activation signal. RNO produced a modest (~2-fold) activation of the reporter, which was not modified in cells treated concurrently (30 minutes) with TG4-155 (100 nM). Nevertheless, TG4-155 (10–100 nM) produced graded, parallel, dextral displacements of ONO-AE1-259 E/[A] curves without suppressing Emax (Fig. 4B). Gaddum/Schild analysis indicated that TG4-155 behaved as a simple, competitive antagonist at a homogeneous receptor population with a pKb of 9.01 ± 0.13 (N = 5) (Fig. 4D). TG4-155 (30 nM) did not affect either the p[A]50 or Emax of ONO-AE1-329 (Fig. 4E).

**Effect of Adenovirus-Mediated Overexpression of PKIA on CRE-Dependent Reporter Activation.** In 6 × CRE BEAS-2B cells infected with Ad5.CMV.PKIA (MOI = 25), PGE2-, ONO-AE1-259-, ONO-AE1-329-, and taprostene-induced reporter activation was suppressed by ~>95%. In contrast, cells infected with a control virus (Ad5.CMV.GFP) responded similarly to native, untreated cells (Fig. 5).

**Effect of PDE4 Inhibitors on ONO-AE1-259- and ONO-AE1-329-Induced, GRE Reporter Activation.** The PDE4 inhibitors, RNO (1 μM) and rolipram (10 μM), produced modest (~2-fold) increases in luciferase activity in 6 × CRE BEAS-2B reporter cells. These interventions also raised the upper asymptotes and, in general, displaced to the left ONO-AE1-259 and ONO-AE1-329 E/[A] curves (Fig. 6, A–D; Table 2). In contrast, rolipram and RNO produced sinistral (~2.5-fold) and, approximately, parallel displacements of indacaterol E/[A] curves without changing Emax (Fig. 6, E and F).

**Effect of ONO-AE1-329 on Dexamethasone-Induced, GRE Reporter Activation.** Additional experiments were performed to assess the interaction between ONO-AE1-329 and the glucocorticoid, dexamethasone, on the activity of a 2 × CRE reporter in BEAS-2B cells. These studies were restricted
Fig. 4. Classification of the prostanoid receptors that mediate CRE-dependent reporter activation. Confluent 6 × CRE BEAS-2B cells were pretreated (30 minutes) with vehicle, the EP4-receptor antagonist, L-161,982 (L-161), the EP2-receptor antagonist, TG4-155 (TG4), or the IP-receptor antagonist, Ro3244794 (Ro) at the concentrations indicated. PGE2 (A), ONO-AE1-259 (B and C), ONO-AE1-329 (D and E) and taprostene (F–H) were added for 6 hours and E/[A] curves constructed. The data in (B, D, and F) were subjected to Gaddum/Schild analysis from which pK_B values were derived. RNO (1 μM) was present in the experiment shown in (B) to enhance the effect of ONO-AE1-259. The dashed horizontal lines indicate baseline reporter activity. Data are means ± S.E.M. of N independent determinations.
to the EP$_4$-receptor agonist because PTGER4 was the only mRNA common to all epithelial cell variants that encoded a Gs-coupled prostanoid receptor (Figs. 1 and 2).

Exposure of 2× CRE BEAS-2B reporter cells to dexamethasone (1 μM) produced a 13.9-fold increase in luciferase activity (Fig. 7A). This effect was augmented by ONO-AE1-329 (10 pM–1μM) in a concentration-dependent manner (p[A]$_{50}$= 8.25 ± 0.07, N = 4) and reached a maximum response that was ∼44-fold higher than baseline. Relative to the enhancement of dexamethasone-induced GRE reporter activation by indacaterol (100 nM), ONO-AE1-329 was partial agonist with an intrinsic activity of 0.77 (Fig. 7A). RNO (1 μM; 30 minutes) also enhanced dexamethasone-induced GRE reporter activation (to ∼29-fold) and interacted with ONO-AE1-329 in an apparently synergistic manner. Thus, the potency of ONO-AE1-329 was increased by 15.1-fold (p[A]$_{50}$: 9.43 ± 0.05, N = 4), and the upper asymptote of the E/[A] curve attained a level equivalent to the indacaterol-induced response (Fig. 7A).

In 2× CRE BEAS-2B reporter cells, dexamethasone increased luciferase activity in a concentration-dependent manner (p[A]$_{50}$ = 8.07) (Fig. 7B; Table 3). RNO (1 μM) and ONO-AE1-329 (1 μM) alone and in combination significantly augmented this response by raising the upper asymptote of the dexamethasone E/[A] curves from ∼14- and 28- to 44- and 54-fold, respectively, without affecting potency (Fig. 7B; Table 3). Thus, these cAMP-elevating interventions were “steroid-sparing.” Indeed, dexamethasone, at a concentration of 30 nM (∼[A]$_{50}$), increased luciferase activity by ∼13-fold whereas in the presence of ONO-AE1-329 and RNO in combination, it generated an equivalent response at a concentration that was ∼7.5-fold lower (Fig. 7B).

**Differential Effects of RNO on ONO-AE1-329-Induced CRE and GRE Reporter Activation.** The degree to which the effects of ONO-AE1-329 were potentiated by RNO was disproportionately greater in 2× CRE reporter cells (15.1-fold) than in the corresponding CRE transfectants (2-fold) (cf. Fig. 8). Assuming an affinity ($K_I$) of ONO-AE1-329 for the EP$_4$-receptor of 9.7 nM (Suzawa et al., 2000), this translated into significant differences in the relationships between receptor occupancy and response (Fig. 8). On both reporters, the relationships were curvilinear and deviated from the line of identity (where response is a linear function of occupancy). In the absence of RNO, occupancy-response curves were similar (Fig. 8) whereas in the presence of RNO a much larger “receptor reserve” (i.e., a higher $K_I$/[A]$_{50}$ ratio) existed for the activation of the GRE compared with the CRE reporter (26.1 and 2.9, respectively; Fig. 8). Quantification of this difference in the presence of RNO revealed that on 2× CRE BEAS-2B cells, 1.5%, 3.8%, and 9.6% receptor occupancy was necessary to produce 20%, 50%, and 80% of the maximal response, respectively. In contrast, considerably greater receptor occupancy (9.4%, 36.8%, and 76.5%, respectively) was required in 6× CRE BEAS-2B cells for ONO-AE1-329 to produce equivalent levels of reporter activation (Fig. 8).

**ONO-AE1-329-, RNO-, and Dexamethasone-Induced Gene Expression Changes in BEAS-2B Cells.** RNA-seq of BEAS-2B cells determined that RNO (1 μM; 2 hours) had little or no effect on the baseline expression of a panel of genes known to be induced by cAMP (Greer et al., 2013; Giembycz and Newton, 2014; BinMahfouz et al., 2015; Yan et al., 2018; Joshi et al., 2019). In contrast, ONO-AE1-329 (1 nM and/or 1 μM; 2 hours) significantly upregulated these genes relative to vehicle matched for time, which was potentiated by RNO in an apparently synergistic manner. For 10 of these induced genes (i.e., CD200, CRISPLD2, GAS1, NR4A1, NR4A2, PDE4D, PDK4, PRDM1, RGS2, SOCS3), the RNA-seq results were validated by PCR and expressed relative to GAPDH (Fig. 9A; Fig. 10). Data on the remaining four transcripts (AVPI1, DEPDC7, NR4A3, SLC7A2) were not PCR validated.
because of the robustness of sequencing and are expressed as TPM (Supplemental Fig. 2). RNO and/or ONO-AE1-329 also repressed baseline gene expression in BEAS-2B cells. PCR-validated data for two of these, EGR1 and TXNIP, are shown in Fig. 9A. Both genes were more repressed by RNO and ONO-AE1-329 in combination than by either drug alone, and this interaction appeared to be at least additive (see Supplemental Table 6 for fold changes).

Consistent with its activity on reporter cells (Fig. 3D), ONO-AE1-329 (1 μM) was a partial agonist in BEAS-2B cells when compared with the LABA, vilanterol (100 nM). Moreover, the intrinsic activity of ONO-AE1-329 varied in a gene-dependent manner ranging from 0.40 on PRDM1 to 0.85 on EGR1 (Fig. 9B; Supplemental Fig. 2).

A companion PCR experiment was conducted with BEAS-2B cells to examine the potential interaction between ONO-AE1-329 (±RNO) and a panel of genes also reported to be regulated by dexamethasone (Joshi et al., 2015a,b; Rider et al., 2018) (Fig. 10). In many cases, these stimuli cooperated in an additive or synergistic manner and the magnitudes of responses were often pronounced (Supplemental Table 6B; Supplemental Table 7). An extreme example was PDK4. This gene was induced 2.1-, 1.7-, and 71-fold by ONO-AE1-329, RNO, and dexamethasone, respectively. However, ONO-AE1-329 and dexamethasone in combination upregulated PDK4 by ~205-fold, and this was increased further to 221-fold by RNO (Fig. 10; Supplemental Table 6B). ONO-AE1-329 and dexamethasone also induced CD200, CRISPLD2, GAS1, PRDM1, and RGS2 in an apparently synergistic manner, and four of those gene expression changes were potentiated by RNO (1 μM) (Fig. 10; Supplemental Table 6B). Similarly, EGR1, which was repressed by ONO-AE1-329 was likewise downregulated by RNO and dexamethasone, but, at the concentrations used, no interaction(s) could be discerned (Fig. 10).

**ONO-AE1-329-, RNO-, and Dexamethasone-Induced Gene Expression Changes in HBECs.** In HBECs grown in submersion culture, ONO-AE1-329 (1 μM) and RNO (1 μM) modulated the expression of AVPI1, DEPDC7, EGR1, GAS1, NR4A3, and PDE4D in either an additive or synergistic manner, whereas PDK4, RGS2, and SLC7A2 were not significantly affected (Fig. 11A; Supplemental Table 7). These findings largely agree with gene expression changes produced by ONO-AE1-329 in BEAS-2B cells. In contrast, most of these genes were not modulated by dexamethasone (1 μM); the only
exceptions were \textit{RGS2} and \textit{EGR1}, which were up- and downregulated, respectively. When ONO-AE1-329 and dexamethasone were used in combination (in the absence and/or presence of RNO), the expression of \textit{PDK4} and \textit{SLC7A2} was now significantly upregulated, \textit{GAS1} and \textit{EGR1} were enhanced, whereas the induction of \textit{DEPDC7} was prevented (Fig. 11; Supplemental Table 7). Relative to vilanterol (100 nM), ONO-AE1-329 was a partial agonist with an intrinsic activity that varied in a gene-dependent manner (Fig. 11B).

Comparable studies using HBECs grown at an ALI revealed that ONO-AE1-329 and dexamethasone promoted gene expression changes that did not exactly mirror their genomic effects in HBECs grown in submersion culture (cf. Fig. 11A; Fig. 12A). Thus, whereas \textit{AVPI1}, \textit{EGR1}, and \textit{GAS1} were modulated similarly

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>(p[A]_{50}) (M)</th>
<th>(E_{max}) (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolipram</td>
<td>6</td>
<td>—</td>
<td>1.53 ± 0.3</td>
</tr>
<tr>
<td>ONO-AE1-259</td>
<td>6</td>
<td>7.33 ± 0.23</td>
<td>4.24 ± 0.3</td>
</tr>
<tr>
<td>ONO-AE1-259 + rolipram</td>
<td>6</td>
<td>7.46 ± 0.18</td>
<td>10.2 ± 1.4</td>
</tr>
<tr>
<td>RNO</td>
<td>3</td>
<td>—</td>
<td>1.95 ± 0.2</td>
</tr>
<tr>
<td>ONO-AE1-259</td>
<td>3</td>
<td>7.34 ± 0.18</td>
<td>3.37 ± 0.4</td>
</tr>
<tr>
<td>ONO-AE1-259 + RNO</td>
<td>3</td>
<td>7.24 ± 0.14</td>
<td>8.06 ± 1.5</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4</td>
<td>—</td>
<td>1.58 ± 0.1</td>
</tr>
<tr>
<td>ONO-AE1-329</td>
<td>4</td>
<td>8.16 ± 0.10</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>ONO-AE1-329 + rolipram</td>
<td>4</td>
<td>8.82 ± 0.09(\ast)</td>
<td>15.3 ± 2.2</td>
</tr>
<tr>
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<td>5</td>
<td>—</td>
<td>1.67 ± 0.2</td>
</tr>
<tr>
<td>ONO-AE1-329</td>
<td>5</td>
<td>8.17 ± 0.12</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td>ONO-AE1-329 + RNO</td>
<td>5</td>
<td>8.48 ± 0.09(\ast)</td>
<td>14.5 ± 0.7</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4</td>
<td>—</td>
<td>1.58 ± 0.1</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>4</td>
<td>9.11 ± 0.06</td>
<td>19.2 ± 2.6</td>
</tr>
<tr>
<td>Indacaterol + rolipram</td>
<td>4</td>
<td>9.51 ± 0.08(\ast)</td>
<td>19.3 ± 3.6</td>
</tr>
<tr>
<td>RNO</td>
<td>6</td>
<td>—</td>
<td>1.95 ± 1.2</td>
</tr>
<tr>
<td>Indacaterol</td>
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<td>17.3 ± 1.7</td>
</tr>
<tr>
<td>Indacaterol + RNO</td>
<td>6</td>
<td>9.34 ± 0.12(\ast)</td>
<td>17.8 ± 2.4</td>
</tr>
</tbody>
</table>

\(\ast\)P < 0.05, significantly different from ONO-AE1-259, ONO-AE1-329, or indacaterol alone.
both epithelial cells variants, neither stimulus, either alone or in combination, significantly \( (P, 0.05) \) regulated \( \text{DEPDC7} \), \( \text{NR4A3} \), or \( \text{SLC7A2} \) (Fig. 12A). In contrast, \( \text{PDK4} \) and \( \text{RGS2} \) were upregulated by dexamethasone (as in HBECs grown as monolayers) whereas \( \text{ONO-AE1-329} \) was without effect and did not interact with the glucocorticoid. However, coregulation of \( \text{RGS2} \) and \( \text{PDK4} \) by \( \text{ONO-A1-329} \) and dexamethasone could not be unequivocally excluded given that a sample size of five may lack sufficient power to detect all gene expression changes. This possibility also applies to \( \text{NR4A3} \), which was increased by \( \text{ONO-AE1-329} \) in every determination but did not reach statistical significance (Fig. 12A).

Relative to formoterol (1 nM), \( \text{ONO-AE1-329} \) was a partial agonist on \( \text{EGR1} \) \( (\alpha = 0.91) \) yet had an intrinsic activity of 1.67 on the only other cAMP-sensitive gene studied in ALI cultures, \( \text{AVPII} \) (Fig. 12B).

### Discussion

This study was undertaken to identify the prostanoid receptors on human airway epithelial cells that could impart therapeutic benefit in individuals with asthma, COPD, and IPF by promoting changes in cAMP-dependent gene expression. The results revealed a dominant role of the \( \text{EP4-subtype} \), which responded to a selective agonist, \( \text{ONO-AE1-329} \), by coupling to a canonical, \( \text{Gs} \) \( \text{a}- \) dependent signaling pathway regulated by \( \text{PDE4} \). \( \text{ONO-AE1-329} \) also augmented glucocorticoid-dependent gene expression changes implying...
Fig. 9. ONO-AE1-329–induced gene expression changes in BEAS-2B cells and the effect of RNO. BEAS-2B cells were pretreated (30 minutes) with RNO (1 μM) or vehicle (V) and then exposed to ONO-AE1-329 (1 nM, ONO1; 1 μM, ONO1000) and vilanterol (Vil; 100 nM) for an additional 2 hours. RNA was extracted and reverse transcribed to cDNA, and gene expression was determined by real-time PCR. (A) Box and whisker plots of gene expression changes normalized to GAPDH from N independent determinations each shown as a black circle. Horizontal dashed lines indicate mean gene expression in vehicle-treated cells. Box and whiskers in red show the sum of the effects produced by ONO-AE1-329 and RNO alone (i.e., \( \Sigma \) (ONO1, RNO)-V, \( \Sigma \) (ONO1000, RNO)-V). (B) Bar charts showing the fold change in gene expression produced by ONO1000 and its associated intrinsic activity (\( \alpha \)) relative to vilanterol. Horizontal dashed lines indicate baseline gene expression. Data are means ± S.E.M. of N independent determinations. Fold changes in gene expression relative to vehicle are provided in Supplemental Table 6A. ND, not determined. Data were analyzed by repeated measures, one-way ANOVA followed by Tukey’s multiple comparisons test. \( *p < 0.05 \), significantly different from: V, RNO, ONO1, ONO1 + RNO, \( \Sigma \) (ONO1, RNO)-V, ONO1000, ONO1000 + RNO and \( \Sigma \) (ONO1000, RNO)-V, respectively.
that enhanced anti-inflammatory activity could be realized if these drugs are used in combination. Collectively, the data suggest that genomic mechanisms likely contributed to the efficacy of EP4-receptor agonists in preclinical models of obstructive lung diseases (see Introduction), which we submit could extend to humans.

Expression of Prostanoid Receptor mRNAs. In BEAS-2B cells, transcripts that encode six (EP2, EP3, EP4, FP, IP, TP) prostanoid receptors were identified by RNA-seq, and this was confirmed by TaqMan PCR. Both methods established that mRNAs encoding the EP4, FP, and TP-receptors were the most abundant as in other human airway epithelial cell lines (Tavakoli et al., 2001; Gray et al., 2004; Joy and Cowley, 2008; Scheckenbach et al., 2011). Transcripts for PTGER4 also predominated in HBECs grown in submersion culture and at an ALI, which is consistent with the intense immunostaining for EP4-receptors on bronchial epithelial cells in human lung (www.proteinatlas.org). It is notable, that although RNA-seq detected 99 GPCR mRNAs that were common to HBECs grown in submersion culture and at an ALI, the rank order of abundance was different. Moreover, some transcripts in ALI cultures (e.g., PTGER2) were below the detection threshold in HBECs. Although culture conditions may account for these discrepancies, cell differentiation is an equally compelling explanation. Indeed, logic dictates that some receptors may be only expressed on
highly differentiated or specialized elements of the epithelium such as ciliated or goblet cells.

Identification and Characterization of Gs-Coupled Prostanoid Receptors. In 6 × CRE BEAS-2B reporter cells, PGE2 E/[A] curves were biphasic. In the presence of an EP1-receptor antagonist, L-161,982, the high potency component was displaced to the right whereas the second phase was unaffected. In contrast, the opposite profile was produced when L-161,982 was replaced with an EP2-receptor antagonist, TG4-155. Collectively, these data indicate that PGE2 activated two distinct receptors: initially the EP4-subtype followed progressively by EP2-receptors with increasing concentration. These data are consistent with PGE2 having a high EP2/EP4 Kᵢ ratio (6- to 22-fold) (Abramovitz et al., 2000) and the ability of L-161,982 and TG4-155 in combination to more effectively block

Fig. 11. Interactions between ONO-AE1-329, dexamethasone, and RNO on gene expression in HBECs grown in submersion culture. HBECs were treated and RNA extracted as described in the legend to Fig. 10. The LABA, vilanterol (Vil, 100 nM) was included as a comparator. (A) Box and whisker plots of gene expression changes normalized to GAPDH from N independent determinations, each shown as a black circle. Horizontal dashed lines indicate mean gene expression in vehicle (V)-treated cells. N.B. Box and whiskers in red show the sum of the effects produced by ONO-AE1-329, RNO, and dexamethasone alone [i.e., ∑(ONO, RNO)-V, ∑(Dex, RNO)-V, ∑(Dex, ONO, RNO)-(2V)]. (B) Bar charts showing the fold change in gene expression produced by ONO1000 together with its intrinsic activity (a) relative to vilanterol. Only those genes (6/9) that were significantly regulated by vilanterol are shown. Horizontal dashed lines indicate baseline gene expression. Data are means ± S.E.M. of N independent determinations. Fold changes in gene expression relative to vehicle are provided in Supplemental Table 7. Dex, dexamethasone; ND, not determined. Data were analyzed by repeated measures, one-way ANOVA followed by Tukey’s multiple comparisons test. ∗, ** p < 0.05, significantly different from: V, RNO, Dex, ONO + RNO, ∑(ONO, RNO)-V, ∑(Dex, RNO)-V, ∑(Dex, ONO)-V, ∑(Dex, ONO, RNO)-(2V).
PGE₂-induced CRE reporter activation than either antagonist alone.

The use of selective ligands verified that EP₂- and EP₄-receptors were coupled to CRE reporter activation. ONO-AE1-259 and ONO-AE1-329 (EP₂- and EP₄-receptor agonists, respectively) increased luciferase activity in a concentration-dependent manner. In contrast, ONO-AE-248 (an EP₃-receptor agonist) was inactive despite reports that EP₃B-, EP₃C-, and EP₃D-splice variants can couple to Gs (Namba et al., 1993; Negishi et al., 1995). The likelihood that ONO-AE1-259 and ONO-AE1-329 were acting through their cognate receptors was confirmed by Schild analyses. Thus, the affinities of TG4-155 and L-161,982 reported here ($K_B = 2.7$ nM and 1 nM, respectively) were similar to those determined for the human EP₂-receptor on C6 glioma cells ($K_B = 2.4$ nM) (Jiang et al., 2012) and the human EP₄-receptor on HEK293 cells ($K_I = 2.4$ nM) (Machwate et al., 2001) and human lung macrophages ($K_B = 3.5$ nM) (Gill et al., 2016).

Fig. 12. Interactions between ONO-AE1-329 and dexamethasone on gene expression in HBECs grown at an ALI. Cells were exposed to dexamethasone (Dex; 1 μM), ONO-AE1-329 (ONO; 1 μM), and Dex and ONO in combination for 2 hours. Formoterol (Form, 1 nM) was included as a comparator. RNA was extracted and reverse transcribed to cDNA, and gene expression was determined by real-time PCR. (A) Box and whisker plots of gene expression changes normalized to GAPDH from $N$ independent determinations, each shown as a black circle. Horizontal dashed lines indicate mean gene expression in vehicle (V1)-treated cells. N.B. Box and whiskers in red show the sum of the effects produced by ONO-AE1-329 and dexamethasone alone (i.e., $\Sigma$(ONO, Dex)-V1). (B) Bar charts showing the fold change in gene expression produced by ONO-AE1-329 and its associated intrinsic activity ($\alpha$) relative to the effect of formoterol. Horizontal dashed lines indicate baseline gene expression. Data are means ± S.E.M. of $N$ independent determinations. Fold changes in gene expression relative to V1 are provided in Supplemental Table 8. V2, a second vehicle-treated group in the same cells was included as an internal control. ND, not determined. Data were analyzed by repeated measures, one-way ANOVA followed by Tukey’s multiple comparisons test. $a$–$fP < 0.05$, significantly different from V1, ONO, Dex, ONO + RNO, $\Sigma$(ONO, Dex)-V1, and V2, respectively.
each receptor summate. This pharmacology was replicated with ONO-AE1-259 and ONO-AE1-329, where the upper asymptotes of their respective E/[A] curves were significantly lower than \( E_{\text{m}} \) (defined with the LABA, indacaterol) (Yan et al., 2018). Moreover, at maximally effective concentrations, ONO-AE1-259 and ONO-AE1-329 interacted in a purely additive manner as expected for two partial agonists that generate the same response by activating distinct receptors.

BEAS-2B cells also expressed mRNA that encode IP-receptors. These were defined using taprostene, a stable prostacyclin mimetic, which is selective for the human IP-receptor (Schneider et al., 1993; Jones and Chan, 2005). Consistent with this taxonomy, taprostene-induced CRE reporter activation was blocked by Ro3244794 with a \( K_B \) of 0.7 nM. This affinity is similar to that obtained in CHO-K1 cells expressing the human recombinant IP-receptor (\( K_I = 3.2 \) nM) (Bley et al., 2006) and in BEAS-2B cells expressing the native IP-receptor (\( K_B = 0.6 \) nM) (Wilson et al., 2009). The failure of TG4-155 and L-161,982 to antagonize taprostene-induced responses supports this conclusion.

Taprostene is also reported to upregulate genes in HBECs (33). However, those data cannot be unequivocally ascribed to IP-receptor agonism because the receptor involved was not identified (33). Furthermore, the expression of \( PTGIR \) mRNA in HBECs grown in submersion culture and at an ALI was ∼25-fold lower than the ≥0.25 TPM cutoff level used to define biologically meaningful expression.

Carcinogenesis, Biased Agonism, and System Bias. Overexpression of PKIα in CRE BEAS-2B cells confirmed that PKA mediated reporter activation in response to EP₂-, EP₄-, and IP-receptor agonists signifying a dominant role for Gs-dependent signaling. Nonetheless, prostaglandin receptors can couple to transducers other than, or in addition to, Gs including Gi and β-arrestins (Fujino and Regan, 2006; Leduc et al., 2009; Yokoyama et al., 2013). Activation of these noncanonical mechanisms is associated with dermal and colorectal malignancies (Fujino and Regan, 2003; Buchanan et al., 2006; Chun et al., 2010; Jiang and Deldinede, 2013; O’Callaghan and Houston, 2015), which may explain the reluctance of the pharmaceutical industry to develop selective EP-receptor agonists for obstructive lung diseases (Bhooshan et al., 2016). However, the realization that the EP₂- and EP₄-receptors can interact with multiple effectors also creates an opportunity for drug development by exploiting the phenomenon of biased agonism. Thus, the discovery of agonists that retain clinical benefit while minimizing their ability to promote tumor growth, migration, invasion, and metastases is a credible prospect. Ono Pharmaceuticals recently described a series of unique EP₂-receptor agonists based on a prostacyclin-like scaffold that have high selectivity and intrinsic efficacy for cAMP generation over β-arrestin recruitment (Ogawa et al., 2016a,b). Evidence for biased agonism at the EP₄-receptor is also available (Leduc et al., 2009). If this molecular selectivity is retained in native target tissues, it may be possible to design EP₄-receptor agonists with acceptable therapeutic ratios.

The efficacy of an EP₄-receptor agonist could also be enhanced with a complementary intervention that augments the natural bias of a physiologic system. In Gs-dependent signaling, this can be achieved by “adding on” a PDE inhibitor or by incorporating both pharmacophores in a bifunctional ligand. These approaches have been tested in proof-of-concept experiments with β₂-adrenoceptor agonists and shown to be feasible (Forkuo et al., 2016; Joshi et al., 2017, 2019). The extent to which ONO-AE1-329 displays biased agonism at its cognate receptor is unexplored. Nevertheless, rolipram and RNO potentiated ONO-AE1-329–induced CRE and GRE reporter activation as well as gene expression changes, which confirmed that PDE4 inhibition had skewed the inherent system bias more toward cAMP-dependent signaling.

The potency of ONO-AE1-329 for augmenting dexamethasone-induced GRE reporter activation was increased by RNO to a greater extent than in the equivalent CRE transfectants (~15- vs. 2-fold, respectively). If both cell types are identical, besides the artificial constructs they contain, then could PDE4 inhibition disproportionately increase agonist potency? One explanation is an inherent difference in the amplification factor between the two reporters in response to ONO-AE1-329. The degree of amplification is determined by the mechanism that drives response, and the number and saturauration of signaling elements that constitute the mechanism (Kenakin, 2014). The ability of RNO to augment CRE and GRE reporter activities indicates that these responses are regulated by PDE4. Under normal conditions, PDE4 fine tunes the magnitude and duration of the stimulus. However, if PDE4 is inhibited, the intracellular level of cAMP (i.e., the quantity of stimulus that activates the next, downstream step) will rise. If more cAMP-dependent saturable steps are required to augment CRE reporter activity than to activate their CRE counterparts, then RNO will increase the potency of ONO-AE1-329 disproportionately. This is because signal amplification increases with the number of saturable steps that generate response and the strength of the input stimulus at each step (Kenakin, 2014). Another possibility is that different pools of cAMP activate the CRE and GRE reporters, with PDE4 exerting more stringent control of cAMP hydrolysis in the latter cells. In this scenario, RNO will produce a greater increment in cAMP in the GRE reporters and a more pronounced sinistral displacement of the ONO-AE1-329 E/[A] curve. Regardless of mechanism, these data suggest that PDE4 inhibition may have a greater impact on glucocorticoid-regulated genes whose expression is enhanced by an EP₄-receptor agonist than on genes regulated by cAMP alone. A triple combination therapy may, therefore, impart greater clinical benefit.

EP₄-Receptor–Mediated Gene Expression Changes. ONO-AE1-329 had a significant genomic impact in human airway epithelial cells. Of potential interest was that some genes were affected similarly across all epithelial cell types studied, whereas others were regulated in a variant-dependent manner (cf. Figs. 9–12). These discrepancies may relate to culture conditions or phenotypic variation between primary cells and a cell line. Moreover, as suggested above for GPCR mRNAs, HBECs grown as a monolayer in submersion culture or that have differentiated at an ALI may simply generate different transcriptional signatures. Regardless, many of these cAMP-regulated genes encode proteins with therapeutic activity (Giembycz and Newton, 2014; Yan et al., 2018). Examples include CD200, NR4A1, NR4A2, NR4A3, SLC7A2, and SOCS3, which modulate inflammatory processes (Yoshimura et al., 2007; Snelgrove et al., 2008; Rodriguez-Calvo et al., 2017; Coburn et al., 2019); a GTPase-activating protein, RGS2, which terminates signaling mediated by Gq-coupled
receptors (Holden et al., 2011, 2014); proteins involved in cell cycle arrest such as DEPDC7 and GAS1 (Del Sal et al., 1992; Samuelsson et al., 1999; Liao et al., 2017); a lipopolysaccharide-binding protein, CRISPLD2, which can neutralize the pathogenicity of Gram negative bacteria (Wang et al., 2009; Zhang et al., 2016); and EGR1 and TXNIP, which are implicated in the etiology of airway fibrosis and oxidative stress, respectively (Nishiyama et al., 1999; Lee et al., 2004; Cho et al., 2006), but were repressed by ONO-AE1-329. Dexamethasone regulated many of these same genes and, moreover, often cooperated with ONO-AE1-329 and/or RNO in an additive or synergistic manner. Although it can be challenging to directly assign a change in gene expression to a particular functional outcome, data are available. For example, in human ASM the cAMP-dependent induction of RGS2 attenuates the magnitude of Ca^{2+} mobilization mediated by Gq-coupled receptors (Holden et al., 2011). In mice, this genomic effect is enhanced by glucocorticoids and protects against agonist-induced bronchoconstriction (Holden et al., 2011).

cAMP-generating stimuli also increase the expression of adverse-effect genes (Tavakoli et al., 2001; Li et al., 2011; Akaba et al., 2018; Yan et al., 2018) although these changes are often moderated or even abolished in cells treated concurrently with a glucocorticoid (Mostafa et al., 2020). This protective interaction has therapeutic implications for using an EP_{2}-receptor agonist with an ICS because ICS/LABA combination therapy often controls individuals with moderate-to-severe asthma in whom an ICS alone does not (Giembycz and Newton, 2015; Newton and Giembycz, 2016).

ONO-AE1-329 was typically a partial agonist on gene expression changes with intrinsic activities relative to vilanterol and formoterol that varied in a transcript-dependent manner. These data suggest that genes of the EP_{2}-receptor–regulated transcriptome interpret equivalent degrees of receptor occupancy differently. This may relate to alternative signaling processes and/or variability in promoter context, which may differentially affect the ability of transcription factors to modulate gene expression. It also implies that the potency of ONO-AE1-329 may vary between genes as reported for β_{2}-adrenoceptor agonists (Joshi et al., 2019).

The genomic activity of ONO-AE1-329 was potentiated by the PDE4 inhibitor, RNO, indicating a central role for cAMP in mediating gene expression changes. The ability of PKIα to abolish ONO-AE1-329–induced CRE reporter activation in BEAS-2B cells provides additional support for this conclusion. It is noteworthy that on most of the genes studied, RNO was inactive. Similar results have been reported previously and were explained by low, basal adenylyl cyclase activity such that a large sample size is required to generate sufficient statistical power to detect modest changes in gene expression (Joshi et al., 2019). Still, a PDE4 inhibitor should exert genomic effects in vivo through its ability to potentiate the tonic activation of Gs-coupled receptors by omnipresent endogenous agonists.

Why an EP_{2}-Receptor Agonist Could Be an Effective Alternative to a LABA. Agonist-driven, cAMP signaling is a compartmentalized phenomenon (Johnstone et al., 2018). Accordingly, unique, gene expression profiles may be generated in the same cell type that depend on the GPCR activated and adenylyl cyclase isofrom to which it couples (Bogard et al., 2014). For the same reason, the profile of gene expression changes could differ in the presence of a glucocorticoid. Thus, a comparative analysis of transcriptomes regulated by EP_{2}-receptor and β_{2}-adrenoceptor agonists (in the absence and presence of a glucocorticoid) could be instructive and is a focus of a current, follow-up investigation. In addition, although detailed mapping of EP_{2}-receptor density and coupling efficiency to adenylyl cyclase in structural, resident, and inflammatory cells in the human lung is lacking, they are not identical to the β_{2}-adrenoceptor. For example, in ALI cultures the PTGER4/ADRB2 mRNA ratio was 4.95 (this study) whereas in human ASM cells, lung fibroblasts, and alveolar macrophages the ratio is 2.8, 0.5, and 7.0, respectively (Einstein et al., 2008; Groot-Kormelink et al., 2012; Roberts et al., 2018). If these relative differences are preserved at the protein level, functional consequences are likely. This is because the Mass Action equation, which underpins receptor theory, predicts that differences in receptor density and/or G-protein coupling efficiency will influence agonist potency and/or the magnitude of response. There may also be variations in the extent to which a given GPCR is subject to agonist-induced desensitization. Together, these factors could influence the therapeutic activity and adverse-effect profiles of agonists that share an ability to stimulate adenylyl cyclase, but act through different GPCRs.

Conclusions

Using human airway epithelial cells as a therapeutically relevant drug target, evidence is presented that an EP_{2}-receptor agonist, alone and in combination with a PDE4 inhibitor and/or a glucocorticoid, may have utility in treating obstructive lung diseases through its ability to induce or repress genes with disease-modifying potential. Indeed, an in vivo genomic effect is predicted throughout airway immune and structural cells that would complement the bronchodilator activity of an EP_{2}-receptor agonist (Buckley et al., 2011). However, the transcriptomic signatures of such ligands in human target tissues harvested from normal individuals and those with obstructive lung diseases need to be defined and possible therapeutic implications investigated. Nevertheless, on the basis of this initial, exploratory, study we submit that asthma, COPD, and IPF be added to the growing list of disorders in which the EP_{2}-receptor could be exploited to therapeutic advantage (Markovic et al., 2017).

Authorship Contributions

Participated in research design: Giembycz, Hamed, Newton, Joshi, Yan.
Conducted experiments: Hamed, Joshi, Michi, Newton, Wiehler, Yan.
Performed data analysis: Giembycz, Hamed, Joshi, Mostafa, Newton, Yan.
Wrote or contributed to the writing of the manuscript: Giembycz, Hamed, Joshi, Michi, Mostafa, Newton, Yan.

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