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Resolvin D1, but not Resolvin E1, Transactivates the Epidermal Growth Factor Receptor to Increase Intracellular Calcium and Glycoconjugate Secretion in Rat and Human Conjunctival Goblet Cells

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Abstract

Purpose: To identify interactions of the epidermal growth factor receptor (EGFR) with the pro-resolving mediator receptors for RvD1 and RvE1 to stimulate an increase in intracellular $[Ca^{2+}]_i$ and mucin secretion from cultured human and rat conjunctival goblet cells.

Methods: Goblet cells from human and rat conjunctiva were grown in culture using RPMI media. Cultured goblet cells were pre-incubated with inhibitors, and then stimulated with RvD1, RvE1, EGF or the cholinergic agonist carbachol (Cch). Increase in $[Ca^{2+}]_i$ was measured using fura-2/AM. Goblet cell secretion was measured using an enzyme-linked lectin assay with UEA-1. Western blot analysis was performed with antibodies against AKT and ERK 1/2.

Results: In cultured human conjunctival goblet cells RvE1 -stimulated an increase in $[Ca^{2+}]_i$. The RvD1-, but not the RvE1-, stimulated increase in $[Ca^{2+}]_i$ and mucin secretion was blocked by the EGFR inhibitor AG1478 and siRNA for the EGFR. RvD1-, but not RvE1-stimulated an increase in $[Ca^{2+}]_i$ that was also inhibited by TAPI-1, an inhibitor of the matrix metalloprotease ADAM 17. Inhibition of the EGFR also blocked RvD1-stimulated increase in AKT activity and

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both RvD1- and RvE1-stimulated increase in ERK 1/2 activity. Pretreatment with either RvD1 or RvE1 did not block the EGFR-stimulated increase in $[Ca^{2+}]_i$.

Conclusions: We conclude that in cultured rat and human conjunctival goblet cells, RvD1 activates the EGFR, increases $[Ca^{2+}]_i$, activates AKT and ERK1/2 to stimulate mucin secretion. RvE1 does not transactivate the EGFR to increase $[Ca^{2+}]_i$ and stimulate mucin secretion, but does interact with the receptor to increase ERK 1/2 activity.

1. Introduction:

The ocular surface comprises the cornea, conjunctiva and its overlying tear film. This tear film is complex, composed of multiple layers secreted by both glands and ocular tissues (Dartt 2004). The innermost layer, the mucous layer, consists of secreted mucins, electrolytes, and water produced by conjunctival goblet cells. Conjunctival goblet cells produce the large gel-forming mucin, MUC5AC, in response to a multitude of stimuli, including epidermal growth factor (EGF) (Hodges et al. 2012; Inatomi et al. 1997; Jumblatt, McKenzie, and Jumblatt 1999). Mucins, such as MUC5AC, are crucial for maintaining ocular surface homeostasis through hydration and lubrication. Impaired mucin secretion can contribute to a variety of ocular surface diseases including dry eye (Inatomi et al. 1997).

Dry eye disease is a chronic, multi-factorial condition of high prevalence across the Western World. This sight-changing, debilitating condition was recently reported to affect as much as 33% of the adult population worldwide (The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007) 2007; Shimmura, Shimazaki, and Tsubota 1999), with an associated economic cost of \$3.84 billion in the US alone (McDonald et al. 2016). Despite this, there are few effective treatments. Dry eye disease is also secondary to the use of many medications such as epidermal growth factor receptor (EGFR) inhibitors which has recently been documented with their increasing use in the treatment of many cancers (Zhang, Basti, and Jampol 2007; Galimont-Collen et al. 2007; Fraunfelder and Fraunfelder 2012; Eaton et al. 2015). Specifically, a significant number of patients suffer from an evaporative form of dry eye shortly after starting therapy.

In the past decade the discovery of specialized pro-resolving mediators (SPMs), including resolvins (Rv), has opened a new therapeutic approach for ocular surface disease. These lipid molecules have a critical role in actively resolving an acute inflammatory response, such as the uncontrolled inflammation central to dry eye disease. Two Phase II clinical trials using the RvE1 analog RX-10045 to treat dry eye disease have been completed with the positive results for 232 patients reported ('Resolvix announces positive data — Phase 2 trial of resolvix RX-10045 for dry eye syndrome' 2009). RvE1 reduces corneal epithelial barrier disruption and protects against goblet cell loss (de Paiva et al. 2012). Our group showed that both RvD1 and RvE1 stimulate high-molecular weight glycoconjugate secretion in cultured rat goblet cells (Lippestad et al. 2018, 2017) while RvD1 stimulates secretion from human conjunctival goblet cells (Li et al. 2013). RvD1 acts through its Gprotein coupled receptors GPR32 (in humans) and ALX/FPR2 (in rats and humans) (Chiang and Serhan 2017). In contrast, RvE1 acts through BTL1 and ChemR23 (Chiang and Serhan 2017). All these receptors are found in cultured conjunctival goblet cells. (Li et al. 2013; Hodges et al. 2016)

(Hodges et al. 2017). Both RvD1 and RvE1 increase intracellular $[Ca^{2+}]_i$, activate extracellular regulated kinase (ERK1/2), and consequently, regulate mucin secretion (Lippestad et al. 2017, 2018). Hence RvD1 and RvE1 could help maintain the ocular surface and tear film in health.

The EGF family of ligands (EGF, heparin binding-EGF (HB-EGF), transforming growth factor α (TGF- α), and amphiregulin) interacts with four related receptor tyrosine kinases known as ErbB receptors, EGFR (ErbB1), ErbB2 (Her/neu), ErbB3, and ErbB4. All these receptors are expressed in cultured rat conjunctival goblet cells (Gu et al. 2008). When EGF binds to its receptor, it forms homo- and hetero-dimers with other family members to recruit adaptor molecules such as phospholipase C (PLC) γ , phosphatidylinositol 3- kinase (PI3K), Shc, and SOS/Grb2 (Carpenter 2000; Gu et al. 2008). These adaptor molecules then initiate signaling cascades including AKT, activated by PI3K, and ERK1/2, activated by multiple pathways, and leading to stimulation of a plethora of cellular processes, including cell proliferation and mucin secretion (Gu et al. 2008).

Closely associated to the EGFR in the cell membrane are matrix metalloproteinases (MMPs), one of which is an inhibitor of disintegrin and metalloproteinase (ADAM) 17. EGF and its family of ligands are synthesized as membrane bound precursors, which are cleaved by ADAM 17 generating the pro- and mature form of EGF (Zunke and Rose-John 2017). This process is known as ectodomain shedding. The shed EGF can bind to EGFRs in an autocrine or paracrine manner. We previously showed that in conjunctival goblet cells, that cholinergic agonists activate the GPCR muscarinic receptors (MAchR) 1, 2, and 3 to trigger ADAM17 that releases EGF by ectodomain shedding from its membrane-bound precursor pro-EGF. The shed EGF binds to the EGFR. Once activated, the EGFR increases intracellular Ca^{2+} and induces ERK1/2 activity to stimulate mucin secretion (Hodges et al. 2012).

Two studies in the cornea demonstrated a positive interaction between EGF and activation of the ALX/FPR2 receptor. First, in the cornea, EGF-stimulated wound healing is mediated by activation of ERK 1/2 which increases the synthesis of lipoxin A_4 which in turn binds to the ALX/FPR2 receptor (Kenchegowda, Bazan, and Bazan 2011). Second, topical application of RvD1 was also showed to activate the ALX/FPR2 receptor enhancing the expression of phosphorylated EGFR leading to regeneration of corneal epithelium in diabetic mice (Zhang et al. 2018) To date no results of an interaction of RvE1 with EGF or its receptor were published.

Because RvD1 and RvE1 activate specific GPCRs and these Rv as well as EGFR activate ERK 1/2, the purpose of the present study was to determine if RvD1 and/or RvE1 stimulate an increase in $[Ca^{2+}]_i$ and mucin secretion by signaling through the EGFR in cultured human and rat conjunctival goblet cells.

2. Materials and Methods

Synthetic RvD1 and RvE1 were purchased from Cayman Chemical, Ann Arbor, MI. Both compounds were dissolved in ethanol as supplied by the manufacturer and were stored at

80°C with minimal exposure to light. AG1478 was purchased from Tocris (Minneapolis, MN), TAPI 2 was purchased from EMD Biosciences (San Diego, CA). Rat EGF were purchased from Peprotech (Rocky Hill, NJ). Fura2/AM was purchased from Invitrogen (Carlsbad, CA). RPMI 1640 media was from Lonza (Walkersville, MD). siRNA and transfection reagents were purchased from Dharmacon (Lafayette, CO). Antibodies against phosphorylated (active) and total AKT were from Cell Signaling Technologies (Danvers, MA) while antibodies against phosphorylated (active) and total ERK 1/2 were from Santa Cruz Biotechnologies (Santa Cruz, CA). Carbachol was from Sigma-Aldrich (St. Louis, MO).

Immediately prior to use, the Rvs were diluted in Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES, 119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, and 5.5 mM glucose [pH 7.45]) to the desired concentrations and added to the cells. The cells were incubated at 37 °C in the dark. Daily working stock dilutions were discarded following each experiment.

2.1 Human Tissue

Human conjunctiva from both sexes was obtained from Eversight (Ann Arbor, MI). Tissue was placed in Optisol media within 18hrs after death.

2.2 Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO₂ for 1 min, decapitated, and the bulbar and forniceal conjunctiva removed from both eyes. All experiments followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and carried out in accordance to the protocols approved by this committee.

2.3 Cell Culture

Goblet cells from human and rat conjunctiva were grown in organ culture as described and extensively characterized previously (Dartt et al. 2011; Garcia-Posadas et al. 2016; Hayashi et al. 2012; Hodges et al. 2012; Hodges et al. 2017; Hodges et al. 2016). The tissue plug was removed after nodules of cells were observed. First passage goblet cells were used in all experiments. The identity of cultured cells was periodically checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin Ulex Europaeus Agglutinin (UEA)-1 (detects goblet cell secretory product) to ensure that goblet cells predominated.

2.4 Reverse Transcriptase (RT)-PCR.

Cultured human goblet cells were homogenized in TRIzol and total RNA isolated according to manufacturer's instructions. One microgram of total RNA was used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). The cDNA was amplified by the polymerase chain reaction (PCR) using primers specific to human EGFR receptor using the Jumpstart REDTaq Readymix Reaction Mix (Sigma-Aldrich, St. Louis, MO) in a thermal cycler (Master Cycler,

Eppendorf, Hauppauge, NY). The primer sequences were generated using PrimerQuest Tool (Integrated DNA Technologies, Skokie, IL). The forward primer sequence was ATG GTC AAG TGC TGG ATG ATA G and reverse primer sequence was CTT GCT GTG GGA TGA GGT ATT. The second forward primer was CCT GGA AGA GAC CTG CAT TAT C and reverse primer was GCC CGT CAG AGT TAT GCT TTA. These primers generated 226 and 427 bp fragments, respectively. β -Actin primers were forward CGT CAT ACT CCT GCT TGC TGA TCC A and the reverse primer was ATC TGG CAC CAC ACC TTC TAC AAT GG CT. The conditions were as follows: 5 min at 95 °C followed by 35 cycles of 1 min at 94°C, 30 s at annealing temperature for 1 min at 72 °C with a final hold at 72 °C for 10 min. Samples with no cDNA served as the negative control. Amplification products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.5 Measurement of $[Ca^{2+}]_i$

Goblet cells were incubated for 1 h at 37 °C with KRB-HEPES with 0.5% BSA containing 0.5 μ M fura-2/AM (Invitrogen, Grand Island, NY), 8 μ M pluronic acid F127, and 250 μ M sulfinpyrazone followed by washing in KRB-HEPES containing sulfinpyrazone. Inhibitors were added for the last 30 min of the fura-2 incubation. Calcium measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells were selected in each experimental condition. The selected cells were followed for the entire experiment and data were collected in real time. Data are presented as the actual $[Ca^{2+}]_i$ with time or as the change in peak $[Ca^{2+}]_i$. Change in peak $[Ca^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[Ca^{2+}]_i$. Although data are not shown, the plateau $[Ca^{2+}]_i$ was affected similarly to the peak $[Ca^{2+}]_i$.

2.6 Measurement of Glycoconjugate Secretion

Cultured goblet cells were serum starved for 2 h before use and then stimulated with either RvD1, RvE1 or EGF in serum-free RPMI 1640 supplemented with 0.5% BSA for 2 hrs. Inhibitors were added 30 min prior to stimulation. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-1. UEA-1 detects high molecular weight glycoconjugates including mucins produced by goblet cells. The media were collected and analyzed for the amount of lectin-detectable glycoconjugates, which quantifies the amount of goblet cell secretion as described earlier (Hodges et al. 2012; Li et al. 2013; Lippestad et al. 2017, 2018; Hodges et al. 2017). Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

2.7 siRNA Experiments for Depletion of EGFR

First passage goblet cells were grown in 24 well plates to 60% confluence. siRNA specific to the EGFR or negative control siRNA, was added at a final concentration of 100 nM in antibiotic-free RPMI 1640 as described previously (Hodges et al. 2017; Li et al. 2012). Media was removed after 18 hours and replaced with fresh, complete RPMI 1640 and incubated for 48 hours before use.

2.8 Western Blot Analysis

The expression of phosphorylated (active) AKT and ERK 1/2 and total AKT and ERK 1/2 was measured by western blot analysis. Primary cultures of rat conjunctival goblet cells were trypsinized and seeded into 6-well plates. Cells were grown to approximately 80% confluency and serum starved for 24 h. For activation of AKT and ERK 1/2, cells were preincubated with AG1478 (10^{-7} M) for 30 min and then incubated with either RvD1 (10^{-8} M) or RvE1 (10^{-9} M) for 5 min. The reaction was stopped by the addition of ice-cold phosphate buffered saline (PBS, 145 mM NaCl, 7.3 mM Na_2PO_4 at pH 7.2). Cells were homogenized in cell lysis buffer (Cell Signaling Technology) and cells were scraped. The homogenates were collected, sonicated, and centrifuged at $14500 \times g$ for 10 min at 4 °C. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and processed for Western blotting as described previously (Hodges et al. 2012; Horikawa et al. 2003; Chen et al. 2006). Antibodies against phosphorylated AKT and ERK 1/2 were used at 1:500 dilution. Total AKT and ERK 1/2 antibodies were used at a dilution of 1:1000. Secondary antibody was used at a dilution of 1:2000, and immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with Image J software (<http://rsbweb.nih.gov/ij/>).

2.9 Statistical Analysis

Results for glycoconjugate secretion were expressed as the fold-increase above basal. Change in calcium concentration was expressed as change above basal, or real-time change in concentration. Results are presented as mean $\pm$ SEM. Data was analyzed by Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Identification of EGFR in Cultured Human Conjunctival Goblet Cells

Earlier studies demonstrated that the EGFR is expressed in rat conjunctival goblet cells both in vivo and in culture (Gu et al. 2008) and that EGF stimulated glycoconjugate secretion (Hodges et al. 2012). To ensure that the EGFR is also expressed in cultured human goblet cells, RT-PCR was performed. Using two different primer sets specific for the EGFR, a single band was obtained for each set (Figure 1) demonstrating that the EGFR is expressed in cultured human goblet cells.

3.2 RvE1 Increases $[\text{Ca}^{2+}]_i$ in Cultured Human and Rat Conjunctival Goblet Cells

We previously showed that RvD1 stimulates an increase in $[\text{Ca}^{2+}]_i$ and glycoconjugate secretion in cultured rat and human goblet cells (Li et al. 2013; Lippestad et al. 2017). RvE1 is also known to increase in $[\text{Ca}^{2+}]_i$ and glycoconjugate secretion in cultured rat goblet cells (Lippestad et al. 2018). However, the effect of RvE1 on cultured human goblet cells is unknown. Therefore, cultured human cells were stimulated with increasing concentrations of RvE1 (10^{-10} - 10^{-7} M) and $[\text{Ca}^{2+}]_i$ measured. RvE1, at all concentrations, increased $[\text{Ca}^{2+}]_i$ (Figure 2A). Peak $[\text{Ca}^{2+}]_i$ was significantly increased by 75 ± 23.0 , 296.5 ± 100.0 , 482.8 ± 62.5 , and 370.6 ± 65.3 nM, at RvE1 10^{-10} , 10^{-9} , 10^{-8} , and 10^{-7} M respectively (Figure

2B). These data indicate that RvE1 is a potent stimulus that increases $[Ca^{2+}]_i$ in both cultured human and rat conjunctival goblet cells

To determine how the magnitude of the RvD1 and RvE1 responses compare to other agonists known to increase $[Ca^{2+}]_i$ in conjunctival goblet cells, rat goblet cells were stimulated with the cholinergic agonist carbachol (Cch, 10^{-4} M) (Hodges et al. 2012), RvD1 (10^{-8} M) or RvE1 (10^{-9} M) and $[Ca^{2+}]_i$ was measured. Cch significantly increased peak $[Ca^{2+}]_i$ by 165.5 ± 35.6 nM. In cells cultured from the same rats, RvD1 significantly increased $[Ca^{2+}]_i$ by 130.3 ± 26.2 nM while RvE1 significantly increased $[Ca^{2+}]_i$ by 248.8 ± 59.6 nM (Figure 2C and D). These data indicate that RvD1 and RvE1 stimulate $[Ca^{2+}]_i$ to a similar extent as Cch but are effective at substantially lower concentrations.

3.3 Inhibition of the EGFR Blocks RvD1, but not RvE1-stimulated, Increase in $[Ca^{2+}]_i$ and Glycoconjugate Secretion in Cultured Rat Conjunctival Goblet Cells

To determine if either RvD1 or RvE1 activate the EGFR, cultured human and rat goblet cells were preincubated with the EGFR inhibitor AG1478 (10^{-7} M) for 30 min prior to stimulation with either RvD1 or RvE1. $[Ca^{2+}]_i$ was then measured. AG1478 added alone was found to have no effect on basal $[Ca^{2+}]_i$ at 10^{-7} M (data not shown). In cultured human goblet cells, RvD1 (10^{-8} M) significantly increased peak $[Ca^{2+}]_i$ by 574.2 ± 145.1 nM (Figure 3A). Preincubation with AG1478 significantly inhibited the RvD1 response and was 100.9 ± 13.1 nM. In cells cultured from the same individuals, RvE1 (10^{-9} M) significantly increased peak $[Ca^{2+}]_i$ by 641.9 ± 23.6 nM (Figure 3A). In contrast to RvD1, AG1478 had no effect on RvE1-stimulated increase in $[Ca^{2+}]_i$ and was 695.1 ± 351.5 nM.

In cultured rat goblet cells, RvD1 significantly increased peak $[Ca^{2+}]_i$ by 473.7 ± 63.5 nM (Figure 3B). This increase was significantly inhibited by AG1478 and was 174.8 ± 63.5 nM. Similar to human cells, RvE1 significantly stimulated an increase in $[Ca^{2+}]_i$ and was 282.4 ± 87.0 nM. This response was not altered by preincubation with AG1478 (Figure 3B). Thus RvD1, but not RvE1, activates the EGFR to increase $[Ca^{2+}]_i$ in both human and rat conjunctival goblet cells.

To determine the effect of AG1478 on RvD1- and RvE1-stimulated glycoconjugate secretion, cultured rat goblet cells were preincubated for 30 min with AG1478 (10^{-7} M) prior to stimulation. RvD1 (10^{-8} M) alone significantly stimulated glycoconjugate secretion 2.4 ± 0.2 while RvE1 (10^{-9} M) increased secretion 2.2 ± 0.1 fold above basal (Figure 4). Similar to the increase in $[Ca^{2+}]_i$, RvD1-stimulated secretion was completely inhibited by AG1478 while RvE1-stimulated secretion was not (Figure 4).

To confirm the results using a chemical inhibitor, we used a second different technique, the use of siRNA to the EGFR. Rat goblet cells were treated with siRNA for EGFR and $[Ca^{2+}]_i$ was measured in response to RvD1 (10^{-8} M), RvE1 (10^{-9} M) or the positive control EGF (10^{-7} M). As shown in Figure 5A, the scrambled siRNA (sc siRNA) had no significant effect on RvD1-stimulated increase in $[Ca^{2+}]_i$ while EGFR siRNA significantly decreased the effect from 399.0 ± 67.4 to 165.7 ± 24.8 nM. In cells cultured from the same rats, RvE1 stimulated $[Ca^{2+}]_i$ to 402.4 ± 16.0 . Neither the sc siRNA nor the EGFR siRNA had any effect on RvE1-stimulated increase in $[Ca^{2+}]_i$. As a control, cells were also stimulated with

EGF (10^{-7} M). EGF increased peak $[Ca^{2+}]_i$ by 511.2 ± 102.9 nM. This increase was significantly inhibited by EGFR siRNA and was 188.6 ± 48.8 nM (Figure 5A).

To verify that the EGFR plays a role in RvD1- and RvE1-stimulated glycoconjugate secretion, rat goblet cells were treated with sc siRNA or EGFR siRNA and glycoconjugate secretion measured. The sc siRNA had no significant effect on RvD1-stimulated glycoconjugate secretion while EGFR siRNA significantly decreased the secretion from 1.6 ± 0.2 to 1.0 ± 0.05 fold above basal (Figure 5B). RvE1 stimulated secretion was unchanged with either sc siRNA or the EGFR siRNA. Again as a control, EGF stimulated glycoconjugate secretion 1.7 ± 0.1 fold above basal. This secretion was significantly inhibited by EGFR siRNA (Figure 5B).

These results demonstrate that RvD1, but not RvE1, interacts with the EGFR on conjunctival goblet cells to increase $[Ca^{2+}]_i$ and stimulate glycoconjugate secretion.

3.4 Inhibition of a MMP Blocks RvD1, but not RvE1-stimulated, Increase in $[Ca^{2+}]_i$ in Cultured Rat Conjunctival Goblet Cells

Activation of the MMP ADAM 17 cleaves membrane bound EGF allowing it to bind to the EGFR on the same or neighboring cells. To understand how RvD1 interacts with the EGFR we used the ADAM 17 inhibitor TAPI-2. In rat conjunctival goblet cells, exposure to TAPI-2 (10^{-6} M) significantly blocked RvD1 (10^{-8} M)-stimulated increase in $[Ca^{2+}]_i$ that was reduced from 301.0 ± 57.5 to 48.6 ± 22.9 nM (Figure 6). There was no inhibition of RvE1 (10^{-9} M)-stimulated increases in $[Ca^{2+}]_i$ by TAPI-2. These results suggest that RvD1, but not RvE1, through activation of the MMP, transactivates the EGFR to increase $[Ca^{2+}]_i$.

3.5 Interaction of ALX/FPR2 Receptors with EGFR in Cultured Rat Conjunctival Goblet Cells

In human conjunctival goblet cells, RvD1 binds to and activates both GPR32 and the ALX/FPR2 receptors. As the GPR32 receptor has not yet been identified in rat tissue (Chiang et al. 2003), it is hypothesized that in this species RvD1 binds to the ALX/FPR2 receptor only. To explore the relationship between ALX/FPR2 and the EGFR, RvD1 was added to rat conjunctival goblet cells followed by EGF 2 min later and $[Ca^{2+}]_i$ was measured (Figure 7A). The order of agonists was then reversed so the EGF was added first and RvD1 added 2 min later. When RvD1 was added first, the peak response increased by 404.8 ± 92.3 nM (Figure 7B). If EGF was added first followed by RvD1, the RvD1 response was significantly decreased to 7.3 ± 3.3 nM. Similar results were obtained with EGF. When EGF was added first, the increase in peak $[Ca^{2+}]_i$ was significantly increased by 359.6 ± 166.2 nM. The EGF response was significantly reduced when added after RvD1 and was 31.6 ± 19.1 nM (Figure 7B). These results suggest that the receptors or signalling pathways activated by the two receptors interact with one another causing this desensitization.

3.6 Inhibition of EGFR Blocks RvD1, but not RvE1, Activation of AKT in Rat Cultured Conjunctival Goblet Cells

In cultured rat conjunctiva activation of the EGFR stimulates a large number of signaling pathways including phosphoinositide-3 kinase (PI-3K) pathway (Prenzel et al. 2001).

Stimulation of the PI-3K pathway leads to phosphorylation of AKT, also known as protein kinase B, and of Shc and Grb2 which increases phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. To determine if RvD1 and RvE1 utilize the EGFR to activate AKT, rat goblet cells were preincubated with AG1478 (10^{-7} M for 30 min prior to stimulation with RvD1 (10^{-8} M) or RvE1 (10^{-9} M) for 5 min. RvD1 significantly increased phosphorylation of AKT 1.4 ± 0.1 fold above basal (Figure 8A and B). Preincubation of cells with AG1478 completely inhibited RvD1-stimulated increase (Figure 8A and B). RvE1 increased phosphorylation of AKT 1.3 ± 0.2 fold above basal. AG1478 did not have any effect on the RvE1-stimulated response (Figure 8 A and B). These data indicate that RvD1 activates the EGFR that in turn activates AKT.

3.7 RvD1 and RvE1 Activate ERK 1/2 in Rat Cultured Conjunctival Goblet Cells

In samples used for AKT, the phosphorylation of ERK 1/2 was determined. RvD1 significantly increased activation of ERK 1/2, 3.1 ± 0.5 fold above basal while RvE1 also significantly increased ERK 1/2 activation 4.9 ± 0.9 fold (Figure 8C and D). In contrast to AKT, preincubation with AG1478 significantly inhibited both RvD1- and RvE1-stimulated activation of ERK 1/2 that was 1.3 ± 0.2 and 1.6 ± 0.4 fold, respectively (Figure 8C and D).

These data indicate that while RvD1 activates the EGFR to stimulate AKT, RvE1 does not. Both Rvs activate the EGFR to stimulate ERK 1/2 although they use different mechanisms.

3.8 Neither RvD1 nor RvE1 Affect EGF-Stimulated Increase in $[Ca^{2+}]_i$ in Cultured Rat Conjunctival Goblet Cells

Previous results demonstrated that both RvD1 and RvE1 counter-regulate the histamine receptors to block histamine-stimulated mucin secretion (Li et al. 2013). To determine if RvD1 and RvE1 also counter-regulate the EGFR, rat goblet cells were preincubated with either RvD1 (10^{-8} M) or RvE1 (10^{-9} M) for 30 min followed by stimulation with EGF (10^{-7}) and $[Ca^{2+}]_i$ measured. As shown in Figure 9, neither RvD1 nor RvE1 had any effect on the EGF response, which was 376.9 ± 31.0 nM. In cells cultured from the same animals, both Rvs significantly reduced the histamine-stimulated response (Figure 9).

These data indicate that RvD1 and RvE1 do not counter-regulate the EGFR.

4. Discussion

In this study, we further identified the signalling pathways used by the pro-resolution mediators RvD1 and RvE1 to stimulate mucin secretion from rat and human conjunctival goblet cells. It is interesting that RvD1 and RvE1 both activate the EGFR but do so by different mechanisms. RvD1 binds to its receptor (ALX/FPR2 in rats), which activates ADAM17 to shed EGF. The released EGF binds to the EGFR to activate AKT and ERK 1/2. In doing so, the two receptors interact with one another to desensitize the other (Figure 10). In contrast, RvE1, after binding to its receptor ChemR23, did not transactivate the EGFR receptor and activate AKT. However, activation of ERK 1/2 by RvE1 is dependent on the EGFR (Figure 10) though it is not clear the mechanism by which this occurs. Possible indirect mechanisms include activation of PLC, PLD or PLA2 as RvE1 induces these enzymes to increase $[Ca^{2+}]_i$ or activation of EGFR adapter proteins not connected to PI3K

including Shc, Grb2, and SOS. An effect similar to that of RvD1 was caused by LTD4 in airway smooth muscle cells (Ravasi et al. 2006). LTD4 using its receptor CysLT1 transactivated the EGFR to stimulate airway smooth muscle cell proliferation, a hallmark of fibrosis in asthma and allergic rhinitis. This pro-inflammatory effect of LTD4 on the EGFR is different than the effect of RvD1 and EGFR in goblet cells that is not associated with inflammation.

In contrast to the differential effect of RvD1 and RvE1 on the EGFR, we previously showed that other signalling pathways activated by RvD1 and RvE1 are similar. Both RvD1 and RvE1 activate phospholipases C, D, and A2 (Lippestad et al. 2017, 2018). Activation of these pathways leads to an increase in $[Ca^{2+}]_i$ and glycoconjugate secretion.

E-series and D-series Rvs have dual anti-inflammatory and pro-resolution roles in maintaining the health of the ocular surface. First, under normal conditions Rvs alone increase $[Ca^{2+}]_i$ and stimulate glycoconjugate secretion to protect the ocular surface and maintain the tear film (Lippestad et al. 2017, 2018). The second role is in disease in which Rvs block different types of inflammatory responses in the ocular surface including histamine and leukotriene (LT)B4 stimulation of mucin secretion to prevent over secretion of mucous (Li et al. 2013; Dartt et al. 2011). Interestingly, when given 30 min before EGF, the Rvs do not alter EGF-stimulated increase in $[Ca^{2+}]_i$ but do inhibit histamine-stimulated increase. This could be because the EGFRs are tyrosine kinases whereas histamine receptors are classical G-protein coupled receptors. The counter-regulation by the Rvs depends on activation of protein kinases that phosphorylate specific sites on the target receptor to down regulate the receptor. Each receptor has specific consensus amino acids that are potential substrates for specific kinases, thus there is inter-receptor variability in the kinases that phosphorylate a given receptor and variability in the kinases activated by the pro-resolution mediator (Hodges et al. 2016; Li et al. 2013). The inter-receptor variability for protein kinase sites predicted from ScanSite4 showed that the human EGFR had 10 predicted motif sites, whereas the human H1 histamine receptor had only two sites neither of which were predicted for the EGFR (<https://scansite4.mit.edu/4.0/#home>). This difference in predicted protein kinase sites is consistent with the inability of RvD1 and RvE1 to counter regulate the EGFR, but the ability to counter-regulate the H1 histamine receptor. The differential down regulation of histamine receptors and EGFR by RvD1 and RvE1 is also consistent with EGF playing a role in health causing goblet cell proliferation and most likely also goblet cell differentiation and MUC5AC synthesis (Takeyama et al. 1999; Hirota et al. 2012), compared to histamine causing inflammation in the conjunctiva.

Our results to date suggest that RvD1 activation of the ALX/FPR2 counter regulates thus inhibiting the GPCRs investigated (Hodges et al. 2016) but does not counter regulate and has no effect on the receptor tyrosine kinases tested. Similar to its effect on the EGFR, RvD1 in rat conjunctival goblet cells does not counter regulate the IL-4, IL5, or IL13 receptors that are used in TH2 allergic inflammatory responses (Garcia-Posadas et al. 2018). In contrast to the lack of effect of RvD1 via ALX/FPR2 on the EGFR in the present study, in HUVEC and mesangial cells LXA₄ using the ALX/FPR2 receptor counter regulates the receptor tyrosine kinases, the VEGFR, PDGFR, and CTGFR thus inhibiting the proinflammatory processes angiogenesis, proliferation and fibrosis (Fierro, Kutok, and Serhan 2002; Wu et al. 2006;

Chiang et al. 2006). Thus, the interaction between RvD1 and ALX/FPR2 with GPCRs and receptor tyrosine kinases are cell, agonist, and receptor (GPCR and RTK) specific.

This study focused on the EGFR as AG1478 is highly specific for the EGFR as well as siRNA directed against the EGFR. The roles of the other ErbB receptors were not investigated. Further work is needed to clarify if the effects seen encompass all ErbB receptors. Resolvins are not immunosuppressant, unlike steroids or other anti-inflammatory agents used in the eye. This is important in the patient group who use EGFR inhibitors as a cancer treatment, as addition of other immune suppressing agents to treat dry eye symptoms could be deleterious to their condition. Use of resolvins could be a novel treatment for cancer (Serhan, Garton, and Panigrahy 2018).

Previous results have demonstrated that cultured rat and human goblet cells react in similar manner. Both RvD1 and RvE1 as well as other pro-resolving mediators, aspirin triggered RvD1 and lipoxin A₄ increase [Ca²⁺]_i and glycoconjugate secretion in both rat and human goblet cells (Hodges et al. 2017; Hodges et al. 2016; Li et al. 2013). Human and rat cultured goblet cells express similar amounts of MUC5AC (Garcia-Posadas et al. 2016). In addition, the responses to interferon γ are similar in both rat and human goblet cells (Garcia-Posadas et al. 2016). As rat goblet cells react very similar to human goblet cells, and have proven to be an excellent model for human goblet cells, the present study is consistent with the similarity of human and rat goblet cells.

The present study uses a cell population that is solely conjunctival goblet cells. In vivo, conjunctival goblet cells are interspersed amongst other cell types including squamous cells and fibroblasts. The dependence and interaction of conjunctival goblet cells on these other populations has not yet been fully elucidated. Furthermore, we do not know the full effect the resolvins may have on these other conjunctival cell populations. Despite these limitations, our in vitro model is a pure population of conjunctival goblet cells, which express all characteristic features of their in vivo counterparts, allowing certainty that all effects seen are specific to goblet cells.

In conclusion, this study demonstrates an important difference for the actions of the D-series compared to the E-series of resolvins in that RvD1, but not RvE1, transactivates the EGFR to stimulate [Ca²⁺]_i and glycoconjugate secretion. In contrast neither Rv counter regulates the EGFR consistent with a role for both the Rvs and the EGFR in maintaining ocular surface health.

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Highlights:

1. RvE1 increases $[Ca^{2+}]_i$ in human conjunctival goblet cells and is potent stimulator in rat conjunctival goblet cells;
2. Inhibition of EGFR blocks RvD1-, but not RvE1-stimulated, increase in $[Ca^{2+}]_i$ and glycoconjugate secretion;
3. Inhibition of MMP blocks RvD1-, but not RvE1-stimulated, increase in $[Ca^{2+}]_i$;
4. Inhibition of EGFR blocks RvD1, but not RvE1, activation of AKT in rat cultured conjunctival goblet cells;
5. Neither RvD1 nor RvE1 affect EGF-stimulated increase in $[Ca^{2+}]_i$ in cultured rat conjunctival goblet cells.

Figure 1. Identification of the EGFR in Cultured Human Goblet Cells.

Human cells were grown, lysed and cDNA generated. RT-PCR was then performed using 2 different primers for the EGFR or the housekeeping gene β -actin. MWM- molecular weight markers. Blot is representative of 3 independent experiments.

Figure 2. RvE1 Increases $[Ca^{2+}]_i$ in Human Conjunctival Goblet Cells and is Potent Stimulator in Rat Conjunctival Goblet Cells.

Cultured human goblet cells were stimulated with RvE1 (10^{-10} - 10^{-7} M) and $[Ca^{2+}]_i$ measured. $[Ca^{2+}]_i$ over time is shown in **A** while change in peak $[Ca^{2+}]_i$ is shown in **B**. Cultured rat cells were incubated with the cholinergic agonist carbachol (Cch, 10^{-4} M), RvD1 (10^{-8} M), or RvE1 (10^{-9} M). $[Ca^{2+}]_i$ over time is shown in **C** while change in peak $[Ca^{2+}]_i$ is shown in **D**. Data are mean $\pm$ SEM from 3–7 (**A** and **B**) or 5 (**C** and **D**) individual experiments. * indicates significant difference from zero.

Figure 3. Inhibition of EGFR Blocks RvD1-, but not RvE1-stimulated, Increase in $[Ca^{2+}]_i$. Conjunctival goblet cells from human (A) and rat (B) were preincubated with the EGFR inhibitor AG1478 (10^{-7} M) for 30 min prior to stimulation with RvD1 (10^{-8} M) or RvE1 (10^{-9} M) and $[Ca^{2+}]_i$ measured. Change in peak $[Ca^{2+}]_i$ is shown. Data are mean $\pm$ SEM from 3 (A) or 4 (B) individual experiments. * indicates significant difference from zero; # indicates significant difference from RvD1 alone.

Figure 4. Inhibition of EGFR Blocks RvD1-, but not RvE1-stimulated, Increase in Glycoconjugate Secretion.

Rat conjunctival goblet cells were preincubated with the EGFR inhibitor AG1478 (10^{-7} M) for 30 min prior to stimulation with RvD1 (10^{-8} M) or RvE1 (10^{-9} M) and glycoconjugate secretion was measured. Data are mean $\pm$ SEM from 3 individual experiments. * indicates significant difference from basal; # indicates significant difference from RvD1 alone.

Figure 5. Inhibition of EGFR Blocks RvD1-, but not RvE1-stimulated, Increase in $[Ca^{2+}]_i$ and Glycoconjugate Secretion.

Rat conjunctival goblet cells were preincubated with the scrambled (sc) siRNA or siRNA against the EGFR prior to stimulation with RvD1 (10^{-8} M), RvE1 (10^{-9} M), or EGF (10^{-9} M) and $[Ca^{2+}]_i$ (A) or glycoconjugate secretion (B) were measured. Data are mean $\pm$ SEM from 4 individual experiments. * indicates significant difference from basal; # indicates significant difference from RvD1 or EGF alone.

Figure 6. Inhibition of MMP Blocks RvD1-, but not RvE1-stimulated, Increase in $[Ca^{2+}]_i$. Rat conjunctival goblet cells were preincubated with the MMP inhibitor TAPI-2 (10^{-6} M) prior to stimulation with RvD1 (10^{-8} M) and RvE1 (10^{-9} M) and $[Ca^{2+}]_i$ measured. Data are mean $\pm$ SEM from 4 individual experiments. * indicates significant difference from zero; # indicates significant difference from RvD1 alone.

Figure 7. Interaction of ALX/FPR2 Receptors with EGFR in Cultured Rat Conjunctival Goblet Cells.

Cultured rat goblet cells were stimulated with either RvD1 (10^{-8} M) or EGF (10^{-9} M) and $[Ca]_i$ measured. Two min later, either RvD1 or EGF was then added. $[Ca]_i$ over time is shown in **A**. Change in peak $[Ca^{2+}]_i$ is shown in **B**. Data are mean $\pm$ SEM from 3–6 individual experiments. * indicates significant difference from zero; # indicates significant difference from RvD1 or EGF alone.

Figure 8. Inhibition of EGFR Blocks RvD1-, but not RvE1-stimulated, Increase in AKT Activation and RvD1 and RvE1-stimulated Increase in ERK 1/2 Activity.

Cultured rat goblet cells were preincubated with AG1478 (10^{-7} M) for 30 min prior to stimulation with either RvD1 (10^{-8} M) or RvE1 (10^{-9} M) for 5 min and western blot analysis was performed using antibodies against phosphorylated and total AKT (A and B), or ERK 1/2 (C and D). Representative blots are shown in A and C. Blots were scanned and data in B and D are mean $\pm$ SEM from 3 individual experiments. * indicates significant difference from basal; # indicates significant difference from RvD1 or RvE1 alone.

Figure 9. RvD1 and RvE1 do not Counter-regulate the EGFR.

Cultured rat goblet cells were preincubated with RvD1 (10^{-8} M) or RvE1 (10^{-9} M) for 30 min prior to stimulation with EGF (10^{-7} M) or histamine (10^{-5} M) and $[Ca^{2+}]_i$ measured. Data are mean $\pm$ SEM from 6 individual experiments. * indicates significant difference from zero; # indicates significant difference from histamine alone.

Figure 10. Schematic Diagram of Signalling Pathways activated by RvD1 and RvE1.

Solid lines indicate known pathways; dotted lines are hypothetical pathways. ADAM 17- an inhibitor of disintegrin and metalloproteinase (ADAM) 17; EGF- epidermal growth factor; EGFR- epidermal growth factor receptor also called ErbB1; Ras- small GTPase; Raf- serine/threonine kinase; MEK- Mitogen-activated protein kinase kinase; ERK 1/2- extracellular signal-regulated kinase 1/2 also known as MAPK; PI3K- phosphatidylinositol4,5-bisphosphate 3-kinase; AKT- serine/threonine kinase also known as protein kinase B; PLC- phospholipase C; PLA₂- phospholipase A₂; PLD- phospholipase D; IP₃- inositol trisphosphate; DAG- diacylglycerol; PKC- protein kinase C; Ca²⁺/CamK- calcium/calmodulindependent protein kinase