Do Cyclosporine A, an IL-1 Receptor Antagonist, Uridine Triphosphate, Rebamipide, and/or Bimatoprost Regulate Human Meibomian Gland Epithelial Cells?

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Purpose. Researchers have hypothesized that treatment with cyclosporine A (CyA), interleukin-1 receptor antagonists (IL-1RA; e.g., anakinra), P2Y2 receptor agonists (e.g., uridine triphosphate; UTP), and rebamipide may alleviate human meibomian gland dysfunction (MGD) and/or dry eye disease. Investigators have also proposed that prostaglandin analogues (e.g., bimatoprost) may induce MGD. Our goal was to determine whether these compounds directly influence human meibomian gland epithelial cell (HMGEC) function.

Methods. Multiple concentrations of each compound were tested for effects on immortalized (I) HMGEC morphology and survival. Nontoxic dosages were used for our studies. Immortalized HMGEC were cultured in the presence of vehicle, CyA, IL-1RA, UTP, rebamipide, or bimatoprost for up to 6 days in various media. Experiments included positive controls for proliferation (epidermal growth factor and bovine pituitary extract), differentiation (azithromycin), and signaling pathway activation (insulin-like growth factor 1). Cells were analyzed for neutral lipid staining, lysosome accumulation, lipid composition, and phosphatidylinositol-3-kinase/Akt (AKT), phosphorylation.

Results. Our findings demonstrate that CyA, IL-1RA, UTP, rebamipide, and bimatoprost had no effect on the proliferation; neutral lipid content; lysosome number; or levels of free cholesterol, triglycerides, or phospholipids in IHMGECs. Cylosporine A, IL-1RA, rebamipide, and bimatoprost significantly reduced the phosphorylation of AKT, as compared to control. Of interest, tested doses of CyA above 8 nM killed the IHMGECs.

Conclusions. Our results show that CyA, IL-1RA, UTP, rebamipide, and bimatoprost do not influence the proliferation or differentiation of IHMGEC. However, with the exception of UTP, these compounds do decrease the activity of the AKT signaling pathway, which is known to promote cell survival.

Keywords: dry eye disease, epithelial cells, human meibomian gland

Dry eye disease (DED) is one of the most frequent reasons for patient visits to eye care practitioners throughout the world, and afflicts over 40 million people in the United States alone.[1–3] Dry eye disease is characterized by a vicious cycle of tear film hyperosmolarity and instability, and leads to increased ocular surface friction, stress, inflammation, and damage, as well as visual impairment.[2,3] The primary cause of DED is obstructive meibomian gland dysfunction (MGD).[4,5] Meibomian gland dysfunction, in turn, is due to hyperkeratinization of the external duct epithelium and a reduced meibum quality (i.e., elevated viscosity), resulting in lipid insufficiency and a heightened evaporation of the tear film.[4,6]

Three drugs have been approved by regulatory agencies in several countries for the treatment of DED. These include cyclosporine A (CyA), an immunosuppressant compound[7]; diquafosol (diuridine-5′-tetraphosphate), a P2Y2 receptor agonist[8]; and rebamipide, a quinolinone derivative.[9] These agents have been reported to alleviate the symptoms and/or signs of DED.[7–26]

In contrast, there is no drug approved for the treatment of MGD. Recently, investigators have proposed that topical therapy with CyA,[27–29] diquafosol,[14,30] or an interleukin-1 receptor antagonist[31] (IL-1RA, anakinra) may be helpful in ameliorating obstructive MGD and its associated evaporative DED. Our goal was to determine whether these compounds directly influence human meibomian gland epithelial cell (HMGEC) function.

Toward that end we examined whether CyA, anakinra, and the P2Y2 receptor agonist uridine-5′-triphosphate (UTP, a diqufosol analogue) regulate the proliferation, differentiation, lipid composition, and signaling in immortalized (I) HMGECs. The secretagogue UTP is analogous to diquafosol and elicits similar actions on the ocular surface.[12,19,22] For comparative purposes, we also tested the effects of rebamipide and bimatoprost on HMGECs. Bimatoprost, a prostaglandin F2α analogue, is an antiglaucoma (i.e., Lumigan) and eyelash lengthening (i.e., Latisse) drug that has been reported to induce MGD and DED.[32–37]

Table. Drugs and Vehicles Used in the IHMGEC Experiments

Drug	Literature Dosage Range	Concentrations Tested in Preliminary Studies	Dose Used for Studies	Drug Vehicle for Cell Cultures
CyA	8 nM–10 μM[8–11]	10 nM, 0.5 μM, 10 μM†	8 nM	0.1% ethanol in medium, 1:10 dilution
UTP*	10–100 μM[6,7]	10, 50, 100 μM	100 μM	Water, 1:10 dilution
Rebamipide	0.01 nM–2 mM[15–18]	1 nM, 1 μM, 1 mM	1 nM	0.01% DMSO in PBS, 1:1000 dilution
IL-1RA	100 ng/mL–500 μg/mL[1–5]	100 ng/mL, 1 μg/mL, 10 μg/mL	10 μg/mL	0.1% BSA in PBS, 1:10 dilution
Bimatoprost	0.01 μM–1.4 mM[12–14]	0.1 μM, 10 μM, 1 mM	10 μM	Ethanol, 1:1000 dilution

DMSO, dimethyl sulfoxide.
* The literature dosage range also includes concentrations for the P2Y2 analogue diquafosol.
† The 10 μM concentration was insoluble and not used.

Materials and Methods

Cell Culture

Immortalized HMGECs were grown in keratinocyte serum-free medium (Life Technologies, Grand Island, NY, USA), as previously described.[38–40] Cells were treated with three doses of CyA, UTP, rebamipide, bimatoprost (all purchased from Santa Cruz Biotechnology, Dallas, TX, USA), or recombinant human IL-1RA (PeproTech, Rocky Hill, NJ, USA). Dosages tested were based upon literature reports evaluating the effects of CyA,[41–44] P2Y2 agonists,[45,46] rebamipide,[47–50] IL-1RA,[51–55] and bimatoprost[56–58] on various primary and immortalized cells (Table). During these preliminary experiments IHMGECs were observed for morphologic changes and cell survival for up to 7 days. Based upon the results of these studies, we then selected the highest concentration for each drug that did not have a dramatic effect on cell survival or morphology. Because all cells were killed by the CyA doses tested, we used the lowest dose found in the literature for subsequent experiments. The following nontoxic doses were selected for all further studies: CyA, 8 nM; UTP, 100 μM; rebamipide, 1 nM; IL-1RA, 10 μg/mL; and bimatoprost, 10 μM (Table). To determine effects of each agent on proliferation, cells were cultured for 6 days with drug or vehicle and counted using a hemocytometer. As a positive control, 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (BPE; Life Technologies) were added to the culture medium.[39]

Lipid Analyses

Differentiation effects were observed in IHMGECs cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; Mediatech, Inc., Manassas, VA, USA), supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 10 ng/mL EGF for 5 days. Cells were exposed to LysoTracker Red DND-99 (Life Technologies) for 30 minutes, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), stained with LipidTOX green neutral lipid stain, and mounted using ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (both from Life Technologies). Slides were viewed using an Eclipse E800 fluorescent microscope and images captured with NIS-Elements Basic Research software, version 4.2 (Nikon Instruments, Melville, NY, USA). Azithromycin (AZM; 10 μg/mL; Santa Cruz Biotechnology) was used as a positive control for the accumulation of neutral lipids and lysosomes in IHMGECs.[59–63]

To identify alterations in specific lipid species, the lipid fraction was isolated from samples containing equivalent cell numbers, as previously described.[61,62] In brief, lipid extracts were developed on a silica gel high-performance thin-layer chromatography (HPTLC) plate (EMD Millipore, Billerica, MA, USA) and compared to cholesterol oleate (Nu-Chek Prep, Elysian, MN, USA), free cholesterol (FC), triolein (both from Sigma-Aldrich Corp., St. Louis, MO, USA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) (all from Avanti Polar Lipids, Alabaster, AL, USA) standards. Nonpolar lipid separation was achieved with benzene:hexane (65:35, vol/vol), while polar lipids were separated using chloroform:methanol:water (65:25:4) followed by benzene:hexane (65:35). Bands were visualized using published techniques.[64] Plates were heated, submerged in acetic acid:sulfuric acid:water (5:0.5:95) with 0.5% $CuSO_4$, then charred. Densitometry calculations on scanned images were performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Azithromycin (10 μg/mL) was utilized as a positive control for the accumulation of free cholesterol and phospholipids, and a reduction in the content of triglycerides, in IHMGECs.[59,61–63]

SDS-PAGE and Immunoblots

Immortalized HMGECs were cultured in DMEM/F12 medium containing 10% FBS for 6 days, then starved in 1% FBS overnight before treatment with drugs or 10 nM recombinant human insulin-like growth factor-1 (IGF-1; National Hormone and Peptide Program, Torrance, CA, USA) for 15 minutes. Subsequently, cells were lysed in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) supplemented with 1% protease inhibitor cocktail, 200 μM sodium orthovanadate, and 5% β-mercaptoethanol (all from Sigma-Aldrich Corp.). Lysates were denatured at 95°C for 10 minutes, separated by SDS-PAGE on 10% Tris/glycine precast gels (Life Technologies), and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibodies specific for phospho-phosphoinositide 3-kinase-protein kinase B (p-AKT) (1:4000, rabbit) or β-actin (1:10,000, mouse; both from Cell Signaling Technology, Danvers, MA, USA), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or Fc-specific goat anti-mouse IgG secondary antibodies, diluted 1:5000 (Sigma-Aldrich Corp.). Blocking and antibody incubation were performed in Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20. Proteins were visualized with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA) using a G-Box gel documentation station (Syngene, Frederick, MD, USA). Image analysis and densitometry were performed using GeneSys software (Syngene). In these studies, IGF-1 served as a positive control.[59]

Results

Influence of CyA, UTP, Rebamipide, IL-1RA, and Bimatoprost on the Proliferation of IHMGECs

To determine whether CyA, UTP, rebamipide, IL-1RA, and bimatoprost modulate the proliferation of IHMGECs, we

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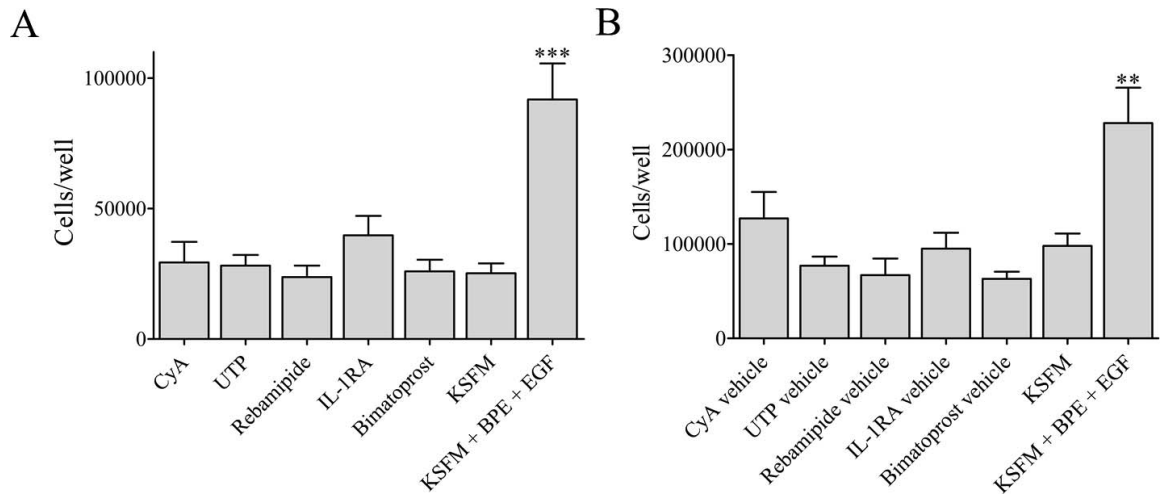

Figure 1. Drugs or vehicles do not alter IHMGEC survival or proliferation. Cells were exposed to treatments or vehicles for 5 days in keratinocyte serum-free medium (KSFM) and counted using a hemocytometer. Cells were exposed to drugs or vehicles at different times under the same conditions. (**A**) Cell counts from three experiments (mean ± standard error) are shown. (**B**) One representative of three control experiments is shown. The combination of epidermal growth factor (EGF, 5 ng/mL) and bovine pituitary extract (BPE, 50 μg/mL) is known to induce IHMGEC proliferation. $^{**}P < 0.01$, $^{***}P < 0.001$, respectively, compared to all other conditions.

cultured cells with these drugs or their vehicles in serum-free media for 5 days ($n = 3$ wells/drug or vehicle/experiment; $n = 3$ experiments/drug). We also compared their effect, if any, to that of EGF plus BPE, a combination known to stimulate IHMGEC proliferation.[39] As shown in Figure 1, and in contrast to EGF plus BPE exposure, neither these drug treatments nor their vehicles had any significant influence on the proliferation of IHMGECs.

Effect of CyA, UTP, Rebamipide, IL-1RA, and Bimatoprost on Lipid and Lysosome Accumulation in IHMGECs

To examine whether CyA, UTP, rebamipide, IL-1RA, and bimatoprost influence lipid and lysosome accumulation in IHMGECs, we treated cells with these drugs, their vehicles, or AZM for 5 days and then processed samples for histologic and biochemical procedures ($n = 3$ wells/treatment/experiment; $n = 3$ experiments/drug). As demonstrated in Figure 2, none of these drugs or their vehicles had any effect on the accumulation of neutral lipids (i.e., LipidTOX staining) or lysosomes (i.e., LysoTracker staining) in IHMGECs. Similarly, these drug and vehicle treatments did not influence the expression of free cholesterol, triglycerides, or phospholipids (Fig. 3). For comparison, AZM increased the appearance of intracellular neutral lipids and lysosomes, elevated the levels of free cholesterol and phospholipids, and reduced the content of triglycerides (Figs. 2, 3).

Impact of CyA, UTP, Rebamipide, IL-1RA, and Bimatoprost on AKT Signaling in IHMGECs

To assess whether CyA, UTP, rebamipide, IL-1RA, and bimatoprost alter the activity of a cell survival mediator, we evaluated whether these drugs influenced AKT signaling. Such a signal, as indicated by AKT phosphorylation, promotes cell growth, proliferation, and survival.[65]

As illustrated in Figure 4, we discovered that CyA, rebamipide, IL-1RA, and bimatoprost significantly reduced the phosphorylation of AKT as compared to control. Uridine triphosphate and the drug-specific vehicles had no effect, whereas IGF-1 significantly increased p-AKT levels (Fig. 4).

Discussion

Our results demonstrate that CyA, IL-1RA, UTP, rebamipide, and bimatoprost have no effect on the proliferation; neutral lipid content; lysosome number; or levels of free cholesterol, triglycerides, or phospholipids in IHMGECs. Further, our data show that CyA, IL-1RA, rebamipide, and bimatoprost significantly decrease the phosphorylation of AKT, a mediator of cell survival, and that tested CyA concentrations above 8 nM kill the IHMGECs. Our findings do not provide any evidence for a positive impact of CyA, IL-1RA, UTP, rebamipide, or bimatoprost on HMGEC function.

Researchers have reported that CyA ameliorates certain signs and/or symptoms of DED.[7,11,24,26] These include improvements in Ocular Surface Disease Index scores, tear film breakup times (TBUT), and Schirmer tests and/or goblet cell densities, as well as a reduction in corneal fluorescein staining.[24,26,66,67] The proposed mechanism(s) of CyA action have ranged from anti-inflammatory effects on the ocular surface,[7] to neuroendocrine influence in the lacrimal gland,[68] to a decrease in action potential generation by corneal cold nerve terminals (Kovacs I, et al. *IOVS* 2012;53:ARVO E-Abstract 1795). Researchers have also hypothesized that CyA treatment may be effective for the therapy of MGD,[27–29] and have found that CyA may reduce the number of orifice inclusions in patients with symptomatic MGD.[28] Given that CyA did not promote the function of HMGECs in our study, it may be that this compound indirectly targets the glandular external duct plugging by suppressing the release of conjunctival proinflammatory mediators that may influence the keratinization process. It is unlikely that CyA does this directly, because this compound is known to induce hyperkeratinization.[69,70]

Investigators have also reported that P2Y2 agonists suppress specific signs and/or symptoms of DED.[8,12,14,19–22] These actions, whether by UTP or diquafosol, are mediated through guanosine triphosphate binding protein-coupled receptors that stimulate goblet cell mucin release and conjunctival chloride transport.[8,12,19,71–77] This latter effect promotes an increase in fluid flow across the conjunctiva and thereby increases tear volume.[8,22,78] The treatment result is a partial or consistent improvement in DED biomarkers, such as ocular surface staining, TBUT, Schirmer test scores, and symptoms.[8,14,21] Because researchers identified P2Y2 recep-

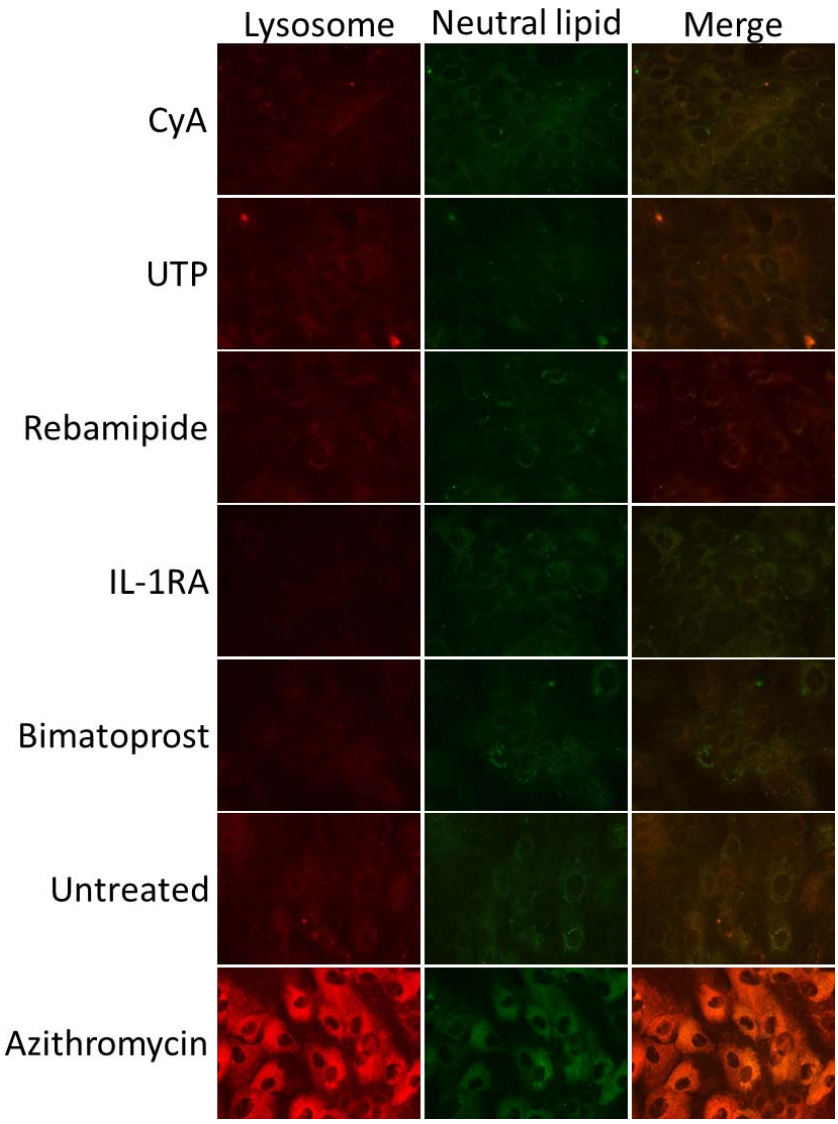

FIGURE 2. Drugs do not alter lipid accumulation or lysosomal expression in IHMGEC. Cells were treated for 5 days in DMEM supplemented with 10% FBS and 10 ng/mL EGF, then stained for lysosomes (LysoTracker Red) and neutral lipid (LipidTOX, *green*). All images ×400 magnification.

tors in ductal epithelial cells of rat, rabbit, and primate meibomian glands,[79,80] a recent study was performed to evaluate topical diquafosol for the treatment of MGD.[30] However, this 4- to 16-month-long study included no placebo-treated controls, which make it impossible to determine whether the P2Y2 agonist elicited an effect in humans. Based upon our findings, if diquafosol does influence the human meibomian gland, the effect might involve ductal, but not acinar, epithelial cells.

In contrast, a recent study with Cu,Zn–superoxide dismutase-1 knockout mice reported that 2 weeks of topical diquafosol treatment increased the number of lipid droplets, the acinar unit density, and keratins 4 and 13 staining in meibomian glands (Ikeda K, et al. *IOVS* 2016;57:ARVO E-Abstract 2869). However, given that this study also contained no placebo-treated controls, it is not clear whether these results are due to diquafosol or the vehicle.

Topical rebamipide has been reported to reduce various signs and/or symptoms of DED.[9,10,13,15–18,23,25] These responses include an increase in mucin expression and optical quality and a reduction in corneal fluorescein staining, ocular surface inflammation, and foreign body sensation.[10,13,16,18,23,81–88] Given that MGD is the most common cause of DED,[4,5] we hypothesized that part of rebamipide's effect could involve promoting meibomian gland function. Our results, though, do not show a stimulatory effect of this quinolinone derivative on HMGECs.

Investigators have hypothesized that topical IL-1RA may have therapeutic benefit as a treatment for desiccating,[89] aqueous-deficient,[90] and MGD-associated evaporative[31] DED. Mouse experiments have shown that IL-1RA may improve ocular surface integrity, increase tear secretion, and suppress corneal inflammation,[89,90] and a human trial discovered that IL-1RA (i.e., anakinra) reduced corneal epitheliopathy in patients with MGD-related DED.[31] However, this latter study did not observe any IL-1RA–associated change in meibomian gland secretion quality or the number of expressible glands as compared to baseline or placebo. This lack of a stimulatory effect of IL-1RA on the meibomian gland was also found in our present study. The rationale for administering IL-1RA is unclear, given that the levels of IL-1RA protein are increased in the tear film of MGD patients with evaporative DED[91] and in the conjunctiva of patients with aqueous-deficient DED.[92]

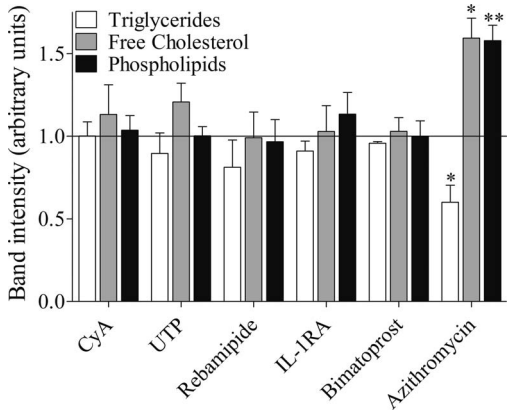

Figure 3. Drugs do not alter the expression of triglycerides, free cholesterol, or phospholipids in IHMGEC. Cells were treated for 5 days in DMEM/F12 supplemented with 10% FBS and 10 ng/mL EGF. The lipid fraction was isolated from samples containing equivalent cell numbers and developed on a silica gel high-performance thin-layer chromatography (HPTLC) plate, compared to lipid standards. Fold change compared to vehicle-treated control cells is shown. Azithromycin (10 μg/mL) is a positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, respectively, compared to control.

Lastly, given that prostaglandins have been reported to induce MGD and DED,[32–37] we hypothesized that bimatoprost, a prostaglandin F2α analogue, may exert a direct action on HMGECs. But like CyA, rebamipide, and IL-1RA, bimatoprost had no effect on the IHMGEC function other than decreasing the phosphorylation of AKT. In contrast, our control compounds, including EGF plus BPE, AZM, and IGF-1, all induced the anticipated alterations in the proliferation; neutral lipid content; lysosome number; levels of free cholesterol, triglycerides and phospholipids; and signaling pathway activity in IHMGECs. Overall, our findings suggest that treatment with

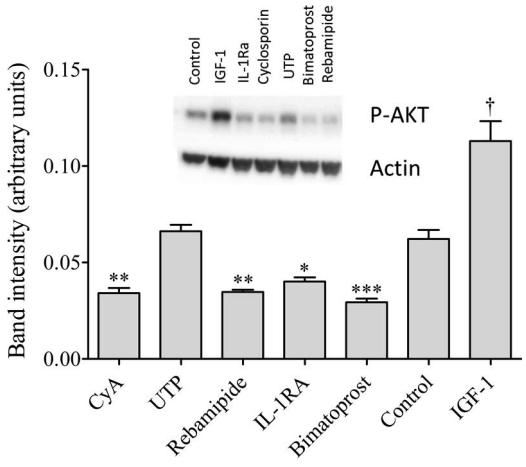

Figure 4. Drugs alter IHMGEC signaling. Cells were cultured for 6 days in DMEM/F12 supplemented with 10% FBS and 10 ng/mL EGF, serum starved overnight (1% FBS), and treated with drugs or vehicles for 15 minutes. Cell lysates were transferred to PVDF and incubated with antibodies specific for phospho-AKT or β-actin. Insulin-like growth factor (IGF-1, 10 nM) is a positive control. Band intensity was normalized to actin and analyzed using ImageJ. By analysis of variance, significant differences exist between groups: $P < 0.0001$. Post hoc analysis using Dunnett's multiple comparisons test indicates that individual treatments significantly decreased ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) or significantly increased ($\dagger P < 0.001$) AKT phosphorylation compared to control.

CyA, IL-1RA, P2Y2 agonists, or rebamipide may not be effective for stimulating the function of HMGECs.

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