Cellular Basis for Bimatoprost Effects on Human Conventional Outflow

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PURPOSE. Bimatoprost is a widely used ocular hypotensive agent to treat glaucoma. It lowers intraocular pressure in humans by increasing both pressure-independent (uveoscleral) and pressure-dependent (conventional) aqueous humor outflow. The present study specifically examines bimatoprost effects on the cells that populate human outflow tissues.

METHODS. The authors tested for prostamide receptor activation in primary cultures of human trabecular meshwork (TM), Schlemm's canal (SC), and ciliary smooth muscle (CSM) cells using cellular dielectric spectroscopy (CDS).

RESULTS. The authors observed that bimatoprost produced an immediate and concentration-dependent increase in cell monolayer impedance for TM, SC, and CSM cells with EC₅₀ values of 4.3, 1.2, and 1.7 nM, respectively; corresponding to decreased cell contractility. Notably, in TM, SC, and CSM cells, bimatoprost was approximately equipotent to the selective FP receptor agonists fluprostenol and 17-phenyl PGF_{2α}. Bimatoprost effects were insensitive to cholera toxin and pertussis toxin but were abolished by phorbol 12-myristate 13-acetate pretreatment, suggesting Gq-involvement in cell signaling. The effects of bimatoprost on TM and SC cells were inhibited by the prostamide receptor antagonist AGN211334, with IC₅₀ values of 1.2 and 3.3 μM, respectively. Interestingly, AGN211334 behaved as an apparent inverse agonist in CDS assays involving TM cells but as a neutral prostamide antagonist with SC cells.

CONCLUSIONS. Taken together, results suggest that bimatoprost specifically activates receptors in both cell types of the human conventional outflow pathway to modify intraocular pressure. However, only TM cell monolayers appear to have autocrine, or agonist-independent, receptor signaling that is sensitive to a prostamide receptor antagonist. (*Invest Ophthalmol Vis Sci.* 2010;51:5176–5181) DOI:10.1167/iops.09-4955

Vision loss associated with glaucoma is treatable if intraocular pressure (IOP) is lowered sufficiently and maintained over time.¹ Because of safety issues, frequency of the dosing

regimen, and their efficacy as ocular antihypertensive agents, prostaglandin (PG)-F_{2α} mimetics have quickly become the medical treatment of choice for managing IOP in glaucoma patients.² PG-F_{2α} drugs lower intraocular pressure by 25% to 30% after 1 week of once-a-day administration without the significant ocular and systemic side effects experienced more frequently with other topical medications.³ Moreover, PGs as a class are much better than other topical glaucoma drugs at controlling diurnal IOP fluctuations, which appear to increase the progression of visual field defects.^{4,5}

Early studies on aqueous humor dynamics in monkeys demonstrated that latanoprost, the prototypical FP receptor agonist, lowered IOP exclusively by increasing uveoscleral outflow.^{6–8} Since these initial reports, recent studies indicate that PGs exert a pronounced effect on pressure-dependent (trabecular/conventional) outflow in human volunteers^{9,10} and in perfused human anterior segments.^{11,12} Although the four PGs currently approved as ocular antihypertensive agents (unoprostone, latanoprost, travoprost, and bimatoprost) do not appear to increase conventional outflow in monkeys, a newer, more potent analog (tafluprost) does, increasing conventional outflow by 33%.¹³ Thus, a class of drugs originally thought to lower IOP solely by effects on uveoscleral outflow clearly impacts conventional outflow.

The chief cellular mechanisms by which PGs exert their effects on uveoscleral outflow appear to be a result of extracellular matrix remodeling between longitudinal ciliary muscle fiber bundles. Alteration in matrix metalloproteinase secretion and opening of flow pathways increase uveoscleral outflow over time.^{14–19} Interestingly, modified flow pathways in chronically treated monkey eyes are lined by endothelial cells, apparently a result of new, organized outflow channel formation between muscle bundles.²⁰ In contrast, the mechanism by which PGs increase conventional outflow in humans is not well understood.

Human trabecular meshwork cells express PG-FP receptors,²¹ and activation of FP receptors in perfused human anterior segments results in both acute (hours) and chronic (days) modulation of outflow facility.^{11,12} The chronic change appears to correspond to the extracellular matrix remodeling observed in the trabecular meshwork (TM) of monkeys treated with PGs for 1 year²⁰ and the altered matrix metalloproteinase secretion detected in cultured human TM cells.¹⁷ The acute increase in outflow facility is consistent with decreased contractility of cultured bovine TM cells.²² Given that previous studies of PG effects on contractility were performed using bovine TM cells and given that Schlemm's canal (SC) cells were not examined, the purpose of the present study was to test the response of PGs on both cell types that populate the human conventional outflow pathway. Using cellular dielectric spectroscopy, we found that bimatoprost significantly increased impedance (an indicator of cell relaxation) of primary cultures of human TM and SC cell monolayers in a receptor-specific

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manner, suggesting that both cell types mediate effects of PGs on conventional outflow facility.

MATERIALS AND METHODS

Cell Culture

Enucleated human donor eyes and eye tissues were obtained from the Life Legacy Foundation (Tucson, AZ), National Disease Research Interchange (Philadelphia, PA), and Sun Health Research Institute (Sun City, AZ). Schlemm's canal (SC) cells were isolated from conventional outflow tissues of whole human eyes or postpenetrating keratoplasty surgical remnants using a cannulation technique and then were characterized and cultured as previously described.²³ Trabecular meshwork (TM) cells were isolated from human eyes using a blunt dissection procedure followed by extracellular matrix digestion and were characterized and cultured as previously described.²⁴ Ciliary smooth muscle (CSM) cells were isolated from fetal human eyes using an explant method previously described.^{25,26} Cell isolates were maintained in Dulbecco's modified Eagle's medium (DMEM; low glucose), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and glutamine (0.29 mg/mL). One CSM strain (CSM2), four different SC cell strains (SC51, SC55, SC58, SC60) and three TM cell strains (TM86, TM90, TM92) were used in the present study. Cells were isolated from eyes of human donors 3 months (TM86), 4 months (TM90), 38 years (TM92), 66 years (SC51), 29 years (SC55), 34 years (SC58), and 58 years (SC60) of age at the time of death.

Chemicals

AGN211334, U46619, and cicaprost were obtained from Allergan (Irvine, CA). Bimatoprost, fluprostenol, and 17-phenyl PGF_{2α} were purchased from Cayman Chemicals (Kalamazoo, MI). Pertussis toxin, cholera toxin, and phorbol 12-myristate 13-acetate were purchased from Sigma-Aldrich (St. Louis, MO).

Cellular Dielectric Spectroscopy

Cellular dielectric spectroscopy (CDS) is a label-free technology based on the measurement of complex impedance changes (ΔZ or dZ). Impedance (Z) is related to the ratio of voltage to current as described by Ohm's law ($Z = V/I$). To measure the changes in impedance that occur in response to receptor stimulation, mammalian cells are seeded onto a custom 96-well microtiter plate that contains electrodes at the bottom of each well. The label-free, cell-based assay system (CellKey; Molecular Devices, Eugene, OR) supplies a constant voltage, producing a current that at low frequencies flows both around and between cells (extracellular current [Ziec]) and at high frequencies flows through cells (transcellular current [Zitc]). On activation of cell surface receptors, signal transduction pathways are initiated, causing morphologic changes. Production of intracellular effectors results in changes in the cellular cytoskeleton that are reflected in changes in the flow of current across and between the cells in the monolayer. This change in the flow of current around and through cells is represented in a single well by an overall change in the impedance of that well. Thus, activation of the cell receptors results in characteristic bioimpedance measurements, indicative of the activation of signaling pathways that are unique to the main subsets of receptors and effectors expressed by that cell type.

For the present study, human cells were seeded onto custom 96-well microplates (CellKey; Molecular Devices) and grown overnight in optimized growth media (DMEM, [low glucose] supplemented with 10% fetal bovine serum, penicillin [100 U/mL], streptomycin [0.1 mg/mL], and glutamine [0.29 mg/mL]) under standard tissue culture conditions. Cell plating density was optimized for each of three cell types used in the present study. Growth media were aspirated, and Hanks HEPES buffer (Invitrogen, Carlsbad, CA) with 0.1% BSA was added to cells to equilibrate for 30 minutes to 1 hour before the start

of each experiment. Equilibrated plates of cells were placed onto the label-free, cell-based assay system (CellKey; Molecular Devices), and baseline measurements were made for 2 minutes at 37°C. Using on-board fluidics, ligands (1 pM–1 μ M) were added to all wells simultaneously while the instrument actively measured the impedance in each well.

Changes in impedance (dZ_{iec}) were captured and quantified in real time for 10 minutes immediately after addition of the drug. Kinetic responses for a selected individual well or all wells were recorded. Extracellular current (dZ_{iec}) responses were quantified by the instrument software by subtraction of the minimum impedance (MIN) reading from the maximum (MAX) impedance reading. To obtain the specific MAX – MIN dZ_{iec} response, dZ_{iec} response in control wells (buffer only) were subtracted from the dZ_{iec} response in wells from which the compound was added. Then, the specific dZ_{iec} was plotted compared with compound concentrations to generate concentration response curves and to calculate EC₅₀/IC₅₀ values. So the dose-response obtained is already the specific impedance changes dZ_{iec} in which the nonspecific impedance changes dZ_{iec} (background) was subtracted. For this study, each graph represents the mean dose-response of three independent experiments using two different cell strains. For comparisons between experiments, we normalized each experiment to bimatoprost (our reference agonist), whose maximum signal was recorded at 10 μ M.²⁷

For competition experiments, cells were pretreated with AGN211334 (10 nM–50 μ M) for 15 minutes before the addition of bimatoprost. For pathway validation studies, cells were treated overnight with pertussis toxin (10 ng/mL), cholera toxin (20 μ g/mL) or phorbol 12-myristate 13-acetate (100 nM) to block Gi or Gs and to or desensitize Gq-mediated signaling pathways, respectively.

Filamentous/Globular Actin Assay

The ratio of filamentous actin (F-actin) to globular actin (G-actin) in two different TM cell strains ($n = 4$) after treatment with drug was determined using a commercially available kit (BK037; Cytoskeleton, Denver, CO). Confluent and mature TM monolayers were serum-starved with HEPES-buffered DMEM for 2 hours and then received 1 μ M bimatoprost or remained untreated for 30 minutes. Cells were then lysed with F-actin stabilization buffer (50 mM PIPES pH 6.9, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β -mercaptoethanol, 0.001% anti-foam C, 1 mM ATP, and Roche protease inhibitor cocktail) and centrifuged (100,000g for 60 minutes at 37°C). Some untreated cell lysates received 1 μ M phalloidin (positive control) before centrifugation. After the supernatant (G-actin) was removed, the pellet (F-actin) was resuspended in an equal volume of ice-cold dH₂O containing 10 μ M cytochalasin-D and kept on ice for 1 hour with mixing every 15 minutes to dissociate the F-actin. An equal volume of G-actin and F-actin samples was then solubilized in 4 $\times$ Laemmli buffer containing 10% β -mercaptoethanol, run on a 10% SDS-polyacrylamide gel, and subjected to immunoblot analysis using standard conditions. The blots were incubated with a rabbit anti-actin antibody (1:500) for 1 hour at 25°C. The relative amounts of F-actin and G-actin were determined by densitometry. Only data in which digitized bands that corresponded to actin bands falling in the linear range of the x-ray film were used for analyses. Values obtained were analyzed by a two-tailed, paired Student's *t*-test assuming unequal variance.

Statistical Analysis

All experiments were performed in duplicate and repeated three times on three different days using two different cell strains. All graphs and data were obtained using computer software (Sigma Plot; Systat Software Inc., San Jose, CA). All the data presented are mean $\pm$ SEM of three independent experiments. Results are expressed as percentage of the bimatoprost activity at a concentration of 10 μ M.

FIGURE 1. Concentration-response relationships for prostacyclin (cicaprost) and thromboxane-A2 (U46619) analogues on monolayer impedance of human outflow cells using cellular dielectric spectroscopy. Effects of increasing concentrations of cicaprost (○) and U46619 (●) were tested on mature monolayers of CSM, TM, and SC cells isolated from human donor eyes. Data shown are mean $\pm$ SEM for three independent experiments using two different cell strains for each cell type.

RESULTS

Control experiments were conducted to examine the range of contractile responses of human ocular cells in culture. Thus, increasing concentrations of two PG agonists, U46619 and cicaprost, working at the TP and IP receptors, respectively, were tested using cellular dielectric spectroscopy. Cell responses were monitored in real time over 10 minutes for each drug concentration on initial exposure. Figure 1 shows the differential responses of ciliary smooth muscle (CSM), trabecular meshwork (TM), and Schlemm's canal (SC) cells. All three cell types contracted (indicated by decreased impedance) robustly when treated with U46619 and had similar EC_{50} s (TM = 7.8 nM; CSM = 12.2 nM; SC = 24.9 nM). Interestingly, SC cells demonstrated the greatest reaction, threefold that of both TM and CSM cells. In contrast, relaxation responses (indicated by increased impedance) of the different cell types to cicaprost treatment were more variable. Cicaprost had very little activity in TM cells while robustly relaxing both CSM and SC cells. Hence, cicaprost had similar EC_{50} s of 1.9 nM and 3.9 nM, respectively, but produced a greater response in SC cells.

Next, the capacity of three PG/prostamide FP receptor agonists to modify the contractility status of human outflow cells was assessed. Shown in Figure 2 are maximal responses of TM, SC, and CSM cells to increasing concentrations of bimatoprost, fluprostenol, and 17-phenyl $PGF_{2\alpha}$. Results show that bimatoprost had an EC_{50} value of 4.3 nM in TM, 1.2 nM in SC, and 1.7 nM in CSM cells. By comparison, fluprostenol had an EC_{50} value of 5.2 nM in TM cells, 5.0 nM in SC cells, and 4.2 nM

in CSM cells, whereas 17-phenyl $PGF_{2\alpha}$ had an EC_{50} of 51 nM in TM cells and 2.8 nM in CSM cells. In SC cells, the EC_{50} values for 17-phenyl $PGF_{2\alpha}$ and bimatoprost were equal at 1.2 nM.

To determine G-protein coupling, cells were first pretreated overnight with pertussis toxin, cholera toxin, or phorbol ester to inactivate G_i , G_s , or G_q , respectively. Figure 3 shows that all three ocular cell types—CSM, TM, and SC—were insensitive to overnight pretreatment with pertussis toxin or cholera toxin; however, preincubation of cells with phorbol ester completely abolished bimatoprost-mediated changes in cellular impedance. Results suggest G_q controls bimatoprost effects on cell relaxation of conventional outflow cells.

We validated CDS findings using a second method that assessed the contractility status of mature TM cell monolayers in culture. Using biochemical methods to monitor actin polymerization, we observed that bimatoprost (1 μ M) decreased the ratio of filamentous actin to globular actin compared with vehicle-treated control (0.61 ± 0.20 vs. 0.79 ± 0.26 [mean $\pm$ SD]; $n = 4$; $P = 0.03$).

Next, increasing concentrations of the prostamide-selective antagonist AGN211334 were applied to cells treated with a single concentration of bimatoprost. Results in Figure 4 show that AGN211334 competitively antagonized bimatoprost effects on cell impedance with IC_{50} values of 3.3 and 1.2 μ M in SC and TM cells, respectively. At concentrations of 10 nM and 100 nM, bimatoprost-mediated effects were completely blocked by AGN211334 in SC and TM cells, respectively. As

FIGURE 2. Concentration-response relationships for bimatoprost, fluprostenol, and 17-phenyl $PGF_{2\alpha}$ on monolayer impedance of human outflow cells using cellular dielectric spectroscopy. Effects of increasing concentrations of bimatoprost (BIM, ▼), fluprostenol (FLU, ▲), and 17-phenyl $PGF_{2\alpha}$ (PGF, ■) were tested on mature monolayers of CSM, TM, and SC cells isolated from human donor eyes. Data shown are mean $\pm$ SEM for three independent experiments using two different cell strains for each cell type.

FIGURE 3. Analyses of G-protein signaling pathway that mediates bimatoprost effects on cell impedance of human outflow cells. Mature monolayers of CSM, TM, and SC cells isolated from human donor eyes were pretreated with cholera toxin (CTX, $\blacktriangle$), pertussis toxin (PTX, $\blacksquare$), or phorbol ester (PMA, $\blacktriangledown$) before exposure to increasing concentrations of bimatoprost (BIM, $\blacklozenge$). Using cellular dielectric spectroscopy, effects of drug combination on cell monolayer impedance was measured. Data shown are mean $\pm$ SEM for three independent experiments using two different cell strains for each cell type.

shown in Figure 5, when applied alone to TM cells, AGN211334 appeared to behave as an inverse agonist, decreasing impedance by approximately 100% compared with baseline. In contrast, AGN211334 behaved as a neutral antagonist and had no discernible effect on baseline impedance values in SC cells.

DISCUSSION

The present study demonstrates that PG effects on conventional outflow facility likely involve receptor activation in both resident cell types, TM and SC cells, of the conventional outflow tissues. Results show that bimatoprost effects were rapid, changing shape/cell-cell contacts between TM and SC cells within seconds and involving the Gq signaling pathway. Although bimatoprost-mediated responses in both TM and SC were completely blocked by the prostamide-specific antagonist AGN211334, treatment with AGN211334 alone resulted in decreased monolayer impedance only in experiments using TM cells. Hence, monolayers of TM but not of SC cells in culture appeared to have an autocrine prostamide receptor-sensitive or constitutive agonist-independent level of signaling.

Because of the tissue architecture, directionality of flow patterns, and proximity of TM and SC cells to each other,

autocrine and paracrine signaling in the conventional outflow pathway represent efficient communication mechanisms to maintain tissue homeostasis. The observation that AGN211334 treatment decreased baseline impedance values in TM cells suggests that only TM cells were actively releasing endogenous prostamide-receptor sensitive compounds under the conditions of the experiments and that the intended targets were autocrine in nature or paracrine (downstream), including cells in the juxtacanalicular tissue/inner wall region. Alternatively, it may be that TM cells have constitutive receptor activity (agonist-independent receptor activity) sensitive to the prostamide antagonist. Regardless, these findings are consistent with a previous report by our laboratory using another, less sensitive model system showing that AGN211334 behaves as an inverse agonist, decreasing hydraulic conductivity of TM cell monolayers.¹¹ To our knowledge these two reports are the only demonstrations of inverse agonism activity (or apparent inverse agonism) of conventional outflow pathway cells.

We chose to include CSM cells in the study as a control because of their documented expression of FP receptors and known involvement in PG/prostamide effects on intraocular pressure, predominantly by way of the uveoscleral pathway. Interestingly, we observed that bimatoprost and the two FP agonists tested relaxed CSM cells. Presumably, cell relaxation of the ciliary muscle results in an immediate but transient increase in uveoscleral outflow. However, the magnitude of such an effect is extremely difficult to assess experimentally in

FIGURE 4. Effects of the prostamide-sensitive receptor antagonist AGN211334 on bimatoprost-mediated stimulation of human outflow cell impedance. Using cellular dielectric spectroscopy, effects of AGN211334 on bimatoprost-mediated stimulation of cell impedance of mature monolayers of human conventional outflow cells were tested. TM and SC cells isolated from human donor eyes were pretreated with increasing concentrations of AGN211334 before exposure to 10 nM and 100 nM bimatoprost, respectively. Data shown are mean $\pm$ SEM for three independent experiments using two different cell strains for each cell type.

FIGURE 5. Concentration-response relationship for AGN211334, a prostamide-sensitive receptor antagonist, on monolayer impedance of human outflow cells using cellular dielectric spectroscopy. Effects of increasing concentrations of AGN211334 on cell impedance of human TM and SC cell monolayers were monitored. Data shown are mean $\pm$ SEM for three independent experiments using two different cell strains for each cell type.

vivo or in situ during this short time frame. Moreover, relaxation of the ciliary muscle should impair the accommodative process, which is much easier to measure. In fact, Kaufman²⁸ predicted our present findings almost 20 years ago after documenting effects of PG F_{2α} isopropyl ester on intraocular pressure, pupil diameter, and accommodation in monkeys.

The functional EC₅₀ values obtained for bimatoprost in TM cells, SC cells, and CSM cells are consistent with the presence of a bimatoprost-sensitive receptor population.^{29–33} Bimatoprost in amide form exhibits no meaningful activity at FP receptors, whereas PGF_{2α} and its anionic analogs, such as 17-phenyl PGF_{2α} and fluprostenol, typically have functional EC₅₀ values in the low nM range.^{29,31–35} Where prostamide activity coexisted with FP receptor expression in the present study, bimatoprost and anionic PGF_{2α} analogs were essentially equipotent^{30–33} when comparing bimatoprost, fluprostenol, and 17-phenyl PGF_{2α} in CSM cells. In TM and SC cells, bimatoprost was actually more potent than 17-phenyl PGF_{2α} and fluprostenol, respectively. In contrast, the functional EC₅₀ values obtained for 17-phenyl PGF_{2α} in the rabbit uterus, feline iris, and human ocular cells were essentially the same.^{29,31} Bimatoprost has, however, been reported as slightly more potent than 17-phenyl PGF_{2α} in the feline lung parenchymal assay.³⁰ The reasons for these differences in potency between cell types and tissues are likely related to their respective PG receptor populations.

The major endogenous PGs are PGD₂, PGE₂, PGF_{2α}, PGI₂, and TxA₂, and each preferentially interacts with receptor subtypes designated DP, EP, FP, IP, and TP, respectively.³⁶ There are subdivisions for DP receptors (DP₁, DP₂) and EP receptors (EP₁, EP₂, EP₃, EP₄). Adding complexity, receptors exist in living cells as dimers, precoupled to heterotrimeric G proteins.³⁷ Receptor dimerization facilitates expansion of the ligand recognition site repertoire, which appears essential for PG receptors to recognize nonenzymatically derived PGs from arachidonic acid³⁸ and the prostamides, which are COX-2-derived products of the endocannabinoid anandamide.³⁹ Thus, 8-epi PGE₂, which is formed by the nonenzymatic oxidation of arachidonate, is recognized by a IP/TP receptor heterodimer.⁴⁰ The bimatoprost-sensitive prostamide receptor is a heterodimer formed between the wild-type FP receptor and an alternative FP mRNA splicing variant.³³

Bimatoprost-sensitive receptors remain to be fully characterized. There are at least six alternative mRNA splicing variants for the FP receptor, and only the altFP4 isoform has been studied to date.³³ Moreover, interactions of bimatoprost with a lipid-sensitive receptor encoded by an entirely different gene cannot yet be ruled out. For example, anandamide was found to be a naturally occurring mammalian ligand for cannabinoid receptors⁴¹ but was later discovered also to stimulate vanilloid receptors.⁴² Thus, mixed interactions of bimatoprost with available target receptors may eventually explain its different functional potencies and tissue specificities, whereas those of 17-phenyl PGF_{2α} remain constant and consistent with interaction with a homogenous receptor population (prostanoid FP).

Our experience shows that the technique of cellular dielectric spectroscopy is a sensitive way to monitor cell relaxation, cell contraction, and changes in cell-cell association to drugs in real time.^{43–45} We were able to obtain full dose-response curves for bimatoprost using CDS compared with only single-point resolution using the ratio of filamentous actin to globular actin and hydraulic contractility of TM cell monolayers. The results of the present study are consistent with those of previous reports demonstrating the importance that relaxation of TM cells plays in increasing conventional outflow facility.^{46–49} Treatment with bimatoprost resulted in an increase in impedance, or a relaxation, whereas treatment with AGN211334 resulted in a decrease in impedance, or a contraction. Inter-

pretation of results with SC cells is more difficult because changes in impedance also reflect changes in cell-cell relationships, a likely component that modulates SC monolayer permeability. Interestingly, responses of SC cells to bimatoprost were consistently more robust than those of TM cells by approximately 15%.

Current strategies to increase outflow facility in humans involve the modulation of TM contractility. Inhibition of intracellular kinases such as ROCK with Y-27632 or sequestration of actin monomers with latrunculin-like compounds are two approaches toward this end. The primary concern with these strategies, however, is the lack of specificity given that all cells in the body have ROCK and actin. In contrast, tissue-specific expression of receptors by TM or SC cells that couple to the contractile machinery (or cell-cell associations) represent an alternative and more viable way to regulate outflow facility and to treat ocular hypertension associated with glaucoma.

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References

1. The Advanced Glaucoma Intervention Study (AGIS), 7: the relationship between control of intraocular pressure and visual field deterioration. The AGIS Investigators. *Am J Ophthalmol*. 2000;130:429–440.
2. Schwartz K, Budenz D. Current management of glaucoma. *Curr Opin Ophthalmol*. 2004;15:119–126.
3. Eisenberg DL, Toris CB, Camras CB. Bimatoprost and travoprost: a review of recent studies of two new glaucoma drugs. *Surv Ophthalmol* 47 Suppl. 2002;1:S105–S115.
4. Bergea B, Bodin L, Svedbergh B. Impact of intraocular pressure regulation on visual fields in open-angle glaucoma. *Ophthalmology*. 1999;106:997–1004, discussion 1004–1005.
5. Asrani S, Zeimer R, Wilensky J, Gieser D, Vitale S, Lindenmuth K. Large diurnal fluctuations in intraocular pressure are an independent risk factor in patients with glaucoma. *J Glaucoma*. 2000;9:134–142.
6. Toris CB, Camras CB, Yablonski ME. Effects of PhXA41, a new prostaglandin F2 alpha analog, on aqueous humor dynamics in human eyes. *Ophthalmology*. 1993;100:1297–1304.
7. Ziai N, Dolan JW, Kacere RD, Brubaker RF. The effects on aqueous dynamics of PhXA41, a new prostaglandin F2 alpha analogue, after topical application in normal and ocular hypertensive human eyes. *Arch Ophthalmol*. 1993;111:1351–1358.
8. Stjernschantz JW. From PGF(2alpha)-isopropyl ester to latanoprost: a review of the development of Xalatan: the Proctor Lecture. *Invest Ophthalmol Vis Sci*. 2001;42:1134–1145.
9. Toris CB, Zhan G, Fan S, et al. Effects of travoprost on aqueous humor dynamics in patients with elevated intraocular pressure. *J Glaucoma*. 2007;16:189–195.
10. Christiansen GA, Nau CB, McLaren JW, Johnson DH. Mechanism of ocular hypotensive action of bimatoprost (Lumigan) in patients with ocular hypertension or glaucoma. *Ophthalmology*. 2004;111:1658–1662.
11. Wan Z, Woodward DF, Cornell CL, et al. Bimatoprost, prostamide activity, and conventional drainage. *Invest Ophthalmol Vis Sci*. 2007;48:4107–4115.
12. Bahler CK, Howell KG, Hann CR, Fautsch MP, Johnson DH. Prostaglandins increase trabecular meshwork outflow facility in cultured human anterior segments. *Am J Ophthalmol*. 2008;145:114–119.
13. Takagi Y, Nakajima T, Shimazaki A, et al. Pharmacological characteristics of AFP-168 (tafluprost), a new prostanoid FP receptor agonist, as an ocular hypotensive drug. *Exp Eye Res*. 2004;78:767–776.
14. Lindsey JD, Kashiwagi K, Boyle D, Kashiwagi F, Firestein GS, Weinreb RN. Prostaglandins increase proMMP-1 and proMMP-3

- secretion by human ciliary smooth muscle cells. *Curr Eye Res.* 1996;15:869–875.
15. Lindsey JD, Kashiwagi K, Kashiwagi F, Weinreb RN. Prostaglandin action on ciliary smooth muscle extracellular matrix metabolism: implications for uveoscleral outflow. *Surv Ophthalmol* 41 Suppl. 1997;2:S53–S59.
 16. Ocklind A. Effect of latanoprost on the extracellular matrix of the ciliary muscle: a study on cultured cells and tissue sections. *Exp Eye Res.* 1998;67:179–191.
 17. Oh DJ, Martin JL, Williams AJ, Russell P, Birk DE, Rhee DJ. Effect of latanoprost on the expression of matrix metalloproteinases and their tissue inhibitors in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2006;47:3887–3895.
 18. Weinreb RN, Lindsey JD, Marchenko G, Marchenko N, Angert M, Strongin A. Prostaglandin FP agonists alter metalloproteinase gene expression in sclera. *Invest Ophthalmol Vis Sci.* 2004;45:4368–4377.
 19. Gaton DD, Sagara T, Lindsey JD, Gabelt BT, Kaufman PL, Weinreb RN. Increased matrix metalloproteinases 1, 2, and 3 in the monkey uveoscleral outflow pathway after topical prostaglandin F(2 α)-isopropyl ester treatment. *Arch Ophthalmol.* 2001;119:1165–1170.
 20. Richter M, Krauss AH, Woodward DF, Lutjen-Drecoll E. Morphological changes in the anterior eye segment after long-term treatment with different receptor selective prostaglandin agonists and a prostamide. *Invest Ophthalmol Vis Sci.* 2003;44:4419–4426.
 21. Anthony T, Pierce K, Stamer D, Regan J. Prostaglandin FP receptors in the human trabecular meshwork. *Invest Ophthalmol Vis Sci.* 1998;39:315–321.
 22. Thieme H, Schimmat C, Munzer G, et al. Endothelin antagonism: effects of FP receptor agonists prostaglandin F2 α and fluprostenol on trabecular meshwork contractility. *Invest Ophthalmol Vis Sci.* 2006;47:938–945.
 23. Stamer WD, Roberts BC, Howell DN, Epstein DL. Isolation, culture and characterization of endothelial cells from Schlemm's canal. *Invest Ophthalmol Vis Sci.* 1998;39:1804–1812.
 24. Stamer WD, Seftor RE, Williams SK, Samaha HA, Snyder RW. Isolation and culture of human trabecular meshwork cells by extracellular matrix digestion. *Curr Eye Res.* 1995;14:611–617.
 25. Hutchinson A, Coons S, Chou C-L, et al. Induction of angiogenic immediate early genes by activation of FP prostanoid receptors in cultured human ciliary smooth muscle cells. *Curr Eye Res.* 2010;35:408–418.
 26. Weinreb RN, Kim DM, Lindsey JD. Propagation of ciliary smooth muscle cells in vitro and effects of prostaglandin F2 α on calcium efflux. *Invest Ophthalmol Vis Sci.* 1992;33:2679–2686.
 27. Peters MF, Knappenberger KS, Wilkins D, et al. Evaluation of cellular dielectric spectroscopy, a whole-cell, label-free technology for drug discovery on Gi-coupled GPCRs. *J Biomol Screen.* 2007;12:312–319.
 28. Crawford KS, Kaufman PL. Dose-related effects of prostaglandin F2 α isopropylester on intraocular pressure, refraction, and pupil diameter in monkeys. *Invest Ophthalmol Vis Sci.* 1991;32:510–519.
 29. Chen J, Senior J, Marshall K, et al. Studies using isolated uterine and other preparations show bimatoprost and prostanoid FP agonists have different activity profiles. *Br J Pharmacol.* 2005;144:493–501.
 30. Woodward DF, Krauss AH, Chen J, et al. Pharmacological characterization of a novel antiglaucoma agent, Bimatoprost (AGN 192024). *J Pharmacol Exp Ther.* 2003;305:772–785.
 31. Woodward DF, Krauss AH, Wang JW, et al. Identification of an antagonist that selectively blocks the activity of prostamides (prostaglandin-ethanolamides) in the feline iris. *Br J Pharmacol.* 2007;150:342–352.
 32. Liang Y, Li C, Guzman VM, et al. Comparison of prostaglandin F2 α , bimatoprost (prostamide), and butaprost (EP2 agonist) on Cyr61 and connective tissue growth factor gene expression. *J Biol Chem.* 2003;278:27267–27277.
 33. Liang Y, Woodward DF, Guzman VM, et al. Identification and pharmacological characterization of the prostaglandin FP receptor and FP receptor variant complexes. *Br J Pharmacol.* 2008;154:1079–1093.
 34. Sharif NA, Kelly CR, Williams GW. Bimatoprost (Lumigan[®]) is an agonist at the cloned human ocular FP prostaglandin receptor: real-time FLIPR-based intracellular Ca(2+) mobilization studies. *Prostaglandins Leukot Essent Fatty Acids.* 2003;68:27–33.
 35. Sharif NA, Williams GW, Kelly CR. Bimatoprost and its free acid are prostaglandin FP receptor agonists. *Eur J Pharmacol.* 2001;432:211–213.
 36. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev.* 1994;46:205–229.
 37. Nobles M, Benians A, Tinker A. Heterotrimeric G proteins pre-couple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci U S A.* 2005;102:18706–18711.
 38. Morrow JD, Minton TA, Mukundan CR, et al. Free radical-induced generation of isoprostanes in vivo: evidence for the formation of D-ring and E-ring isoprostanes. *J Biol Chem.* 1994;269:4317–4326.
 39. Kozak KR, Crews BC, Morrow JD, et al. Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J Biol Chem.* 2002;277:44877–44885.
 40. Wilson SJ, Roche AM, Kostetskaia E, Smyth EM. Dimerization of the human receptors for prostacyclin and thromboxane facilitates thromboxane receptor-mediated cAMP generation. *J Biol Chem.* 2004;279:53036–53047.
 41. Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K, Devane WA. Anandamide, an endogenous cannabinimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc Natl Acad Sci U S A.* 1993;90:7656–60.
 42. Zygmunt PM, Petersson J, Andersson DA, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature.* 1999;400:452–457.
 43. Verdonk E, Johnson K, McGuinness R, et al. Cellular dielectric spectroscopy: a label-free comprehensive platform for functional evaluation of endogenous receptors. *Assay Drug Dev Technol.* 2006;4:609–619.
 44. Atienza JM, Yu N, Wang X, Xu X, Abassi Y. Label-free and real-time cell-based kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. *J Biomol Screen.* 2006;11:634–643.
 45. Yu N, Atienza JM, Bernard J, et al. Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Anal Chem.* 2006;78:35–43.
 46. Peterson J, Tian B, Bershadsky A, et al. Latrunculin-A increases outflow facility in the monkey. *Invest Ophthalmol Vis Sci.* 1999;931–941.
 47. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. *Invest Ophthalmol Vis Sci.* 2001;42:1029–1037.
 48. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. *Exp Eye Res.* 2005;80:215–225.
 49. Ramos RF, Sumida GM, Stamer WD. Cyclic mechanical stress and trabecular meshwork cell contractility. *Invest Ophthalmol Vis Sci.* 2009;50:3826–3832.