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Endogenous Bioactive Lipids and the Regulation of Conventional Outflow Facility

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Summary

Perturbation of paracrine signaling within the human conventional outflow pathway influences tissue homeostasis and outflow function. For example, exogenous introduction of the bioactive lipids, sphingosine-1-phosphate, anandamide or prostaglandin F_{2α}, to conventional outflow tissues alters the rate of drainage of aqueous humor through the trabecular meshwork, and into Schlemm's canal. This review summarizes recent data that characterizes endogenous bioactive lipids, their receptors and associated signaling partners in the conventional outflow tract. We also discuss the potential of targeting such signaling pathways as a strategy for the development of therapeutics to treat ocular hypertension and glaucoma.

Keywords

trabecular meshwork; Schlemm's canal; outflow facility; prostaglandin; prostamide; endocannabinoid; lysophospholipid; glaucoma; intraocular pressure

Introduction

Epidemiology and Pathophysiology of Glaucoma

Glaucoma as a group of diseases is the second leading cause of irreversible blindness in western countries, and affecting approximately 65–70 million people worldwide [1,2]. In the United States epidemiologic data indicates an increasing prevalence of glaucoma over the last decade [3]. Glaucoma is characterized by loss of retinal ganglion cells, degeneration of the optic nerve head and associated visual field loss that begins in the periphery. The majority of glaucoma cases (~70% of total) are classified as “Primary open-angle glaucoma” (POAG) because the angle formed by the cornea and iris is open, and not physically obstructing the outflow pathways. Interestingly, intraocular pressure (IOP) is often abnormally elevated in POAG even though aqueous secretion rate is normal and the conventional outflow pathway is not visibly obstructed either at the light or microscopic levels [4]. Not surprising, the primary risk factor for POAG is elevated IOP (ocular hypertension), which impacts its prevalence, incidence and progression [3]. In addition, other prominent risk factors for POAG include age, African ancestry, corticosteroid use and close relatives with a history of POAG. Less characterized risk factors include diabetes, hypertension and other chronic systemic diseases [5].

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Ocular hypertension associated with POAG is distinguished by decreased movement of aqueous humor through the conventional outflow pathway. Measured by tonography, Grant showed in vivo that glaucomatous eyes have reduced outflow through the conventional tract [6]. Accompanying decreased outflow facility are fewer trabecular meshwork cells in the inner meshwork and more plaque material in the juxtacanalicular tissue (JCT) of the conventional outflow tract [7–9]. Since the conventional outflow pathway is pressure-sensitive and accounts for the majority of aqueous humor outflow (70–90% of total), it is not surprising that cellular dysfunction impacts IOP [10,11].

There are two cell types that populate the conventional outflow pathway. Trabecular meshwork (TM) cells cover the collagen beams as cell monolayers in the uveal and corneoscleral regions (inner meshwork). In contrast, TM cells in the JCT region near Schlemm's canal (SC) do not adhere to a basement membrane; instead are surrounded by extracellular matrix materials. Aqueous humor first passes through the TM before entering SC, a circular vessel that collects and delivers aqueous humor to collector channels which join aqueous and episcleral veins. TM cells on the collagen beams are responsible for removing cellular debris and pigment from aqueous humor before reaching the resistance generating region of the conventional outflow tract, consisting of TM cells in the JCT and the inner wall of SC cells. Resistance generated in this region regulates the volume of aqueous humor per unit time that is driven into SC by a hydrostatic pressure gradient between the inside (IOP) and outside (episcleral venous pressure) of the eye.

Even after many decades of intense research, the precise cellular and extracellular mechanisms that regulate resistance to control IOP in the conventional outflow pathway in normal and glaucomatous eyes remain uncertain. Three main hypotheses have surfaced in the past few years to explain the regulation of resistance in the conventional outflow pathway. Contractility of trabecular meshwork cells, extracellular matrix (ECM) turnover in the JCT and/or permeability of the inner wall of SC are currently thought of as the three primary mechanisms.

Similar to smooth muscle cells, TM cells are contractile, and the level of contractile tone of the trabecular meshwork tissue appears to affect outflow facility. Thus, disrupting actin organization by a variety of drugs increases outflow facility [12–16]. Alternatively, the inhibition of the phosphorylation of myosin light chain kinase with Rho kinase inhibitors also decrease contractility of the trabecular meshwork, and subsequently increase outflow facility [17–19]. Secondly, changes in ECM turnover and remodeling in the JCT region appear to contribute to the regulation of outflow through the conventional tract. For example, a number of treatments known to alter matrix metalloproteinase (MMP) expression/secretion by TM cells change outflow facility [20–22]. Moreover, direct inhibition of MMP activity in the conventional outflow pathway with tissue inhibitors of metalloproteinases or several families of synthetic MMP inhibitors decrease outflow facility [23]. Finally, it appears that the pressure-sensitivity of the conventional tract is mediated by the physical dynamics of the inner wall of SC and the instability of its cell-cell junctions [24,25]. If the openings between inner wall cells are occluded with cationic ferritin, outflow facility dramatically decreases [26]. Interestingly, the number of openings in the inner wall cells is less in glaucomatous eyes than age-matched normal eyes [27–29]. The relative contribution of these three mechanisms (and likely others) to the regulation of outflow facility are currently unknown.

Certain are that effective lowering of IOP in those with POAG is essential in slowing or stopping progression of vision loss (and ganglion cell death). Multiple large clinical trials demonstrate that the amount of vision loss over time is inversely proportional to the level that IOP can be lowered [3]. Interestingly, IOP reduction benefits people with glaucoma whether or not they have elevated IOP. Apparently, IOP lowering below the normal average (i.e. ~12 mmHg) halts disease progression as indicated by stable visual field measurements over time

[30]. Moreover, a recent study has shown that greater IOP fluctuations cause more severe progression of POAG, and possibly a stronger risk factor than mean IOP [31]. Therefore, since the conventional outflow pathway is pressure-sensitive, improving its function will likely effectively lower IOP and limit IOP fluctuations.

Therapeutic Practice, Outcomes, and Limitations

For more than 100 years glaucoma patients have been treated with medication to reduce IOP. Cholinergics were the first class of drugs commonly used to lower IOP. While effective at increasing outflow via the conventional route, side effects including miosis and accommodation have limited daily use. Drugs that lower IOP by inhibition of aqueous humor secretion were subsequently introduced and are still commonly prescribed today [32]. Agents, such as Timolol, decrease aqueous humor secretion and lower IOP by blocking β -adrenoceptors in the ciliary body. In the 1950's a second class of drugs that inhibit aqueous humor secretion was introduced as a systemically administered anti-glaucoma medication, namely the carbonic anhydrase inhibitors. Carbonic anhydrase inhibitors interfere with the formation of bicarbonate, an important molecule involved in fluid movement across the ciliary epithelium. A third class of compounds that target aqueous secretion was launched a little over a decade ago. Adrenergics that activate α_2 -adrenoceptors in the ciliary body are effective at rapidly reducing IOP, but are not routinely used as first-line therapy. The newest class of compounds to lower IOP was introduced in the late 1990's. These new drugs act primarily by activating prostaglandin (PG) FP receptors in the ciliary muscle and increasing uveoscleral outflow [33]. The uveoscleral pathway or unconventional pathway consists of passages for fluid between longitudinal muscle bundles in the ciliary body and accounts for 10–30% of total outflow. Additionally, recent studies show that prostanoid drugs also increase conventional outflow [34,35]. Because of efficacy at lowering IOP in glaucoma patients and minor side effects, prostaglandin analogues today are the first-line choice for the treatment of glaucoma.

Even when multiple IOP-lowering drugs are used in combination, lowering of IOP to a “target pressure” is often not attained, and patients with glaucoma must undergo laser or incisional surgery to effectively lower IOP. To avoid surgery, better medications are needed. Therefore, missing from our armamentarium are drugs that specifically target the conventional outflow pathway. While epinephrine (and cholinergics) effectively increases outflow facility, systemic side effects and difficulties with intraocular delivery have limited daily use. Thus, a major goal of glaucoma researchers today is to identify novel drug targets in the conventional outflow pathway.

Increasing pressure-dependent outflow has several advantages. First, the conventional outflow pathway is the diseased tissue in people with ocular hypertension (and glaucoma) and compensating for the causal molecular defect(s) will likely improve patient outcomes. Second, the conventional outflow tract is the primary pathway for removal of aqueous humor from the eye, thus drugs that increase outflow should have profound effects on IOP. Third, increased perfusion of outflow cells may delay cell loss/drop out that is accelerated in glaucoma. Fourth, increasing pressure-dependent outflow may assist in dampening heightened pressure fluctuations common in some glaucoma patients. Fifth, increasing outflow facility provides a complementary way to decrease IOP for combination therapies. Thus, the purpose of this review is to examine a specific set of novel targets in the conventional outflow pathway; receptors expressed by outflow cells that bind endogenous lipids and appear to function in homeostatic regulation of outflow resistance.

Bioactive Lipids

Prostaglandins

Are PGs capable of influencing outflow facility by targeting conventional drainage tissue directly? It was widely believed, based largely on non-human primate data, that PGs exerted their effects on intraocular pressure exclusively by increasing uveoscleral outflow.

Prostaglandin F_{2α} analogues are the most commonly prescribed anti-glaucoma medication today but their mechanism of action is not fully understood. Latanoprost, being the prototypical FP receptor analog used as a prodrug for treating glaucoma, was originally believed to lower intraocular pressure by increasing uveoscleral outflow with little or no effect on conventional outflow [36–38]. In contrast to latanoprost, travoprost (also a PG F_{2α} analogue) was recently reported to produce a highly significant increase in conventional outflow without a significant effect on uveoscleral outflow; although some effect on uveoscleral outflow cannot be entirely ruled out [39]. The prostamide F_{2α} mimetic, bimatoprost, has been shown to exert a pronounced effect on pressure dependent trabecular outflow in human volunteers [40] and in isolated perfused human ocular anterior segment preparations [34]. Thus, a class of drugs originally thought to solely influence uveoscleral outflow clearly impacts the conventional pathway.

This section will therefore focus on information pertaining to PG mimetics and other drugs known to impact prostaglandin pathways, in order to have a better understanding of the role of endogenous PGs on homeostasis of conventional tissues and subsequent regulation of aqueous humor outflow. We will cover the following topics: I) The presence of endogenous PGs in the conventional outflow pathway; II) How outflow facility and conventional drainage tissue are affected in vivo by PGs; III) The current understanding of the mechanisms responsible for PG-induced intraocular pressure reduction, and how conventional drainage tissue/cells may be involved.

Endogenous Prostaglandins in the Conventional Outflow Pathway

Prostaglandins are produced in various ocular tissues. Steady-state levels of PGD₂, E₂ and F_{2α} and corresponding prostaglandin synthase activities were detected in the ocular tissues [41]. Bioactive agents, laser treatments, and mechanical stress all have effects on endogenous PG synthesis. For example, epinephrine and laser treatment elevate endogenous PG levels in the aqueous humor, while dexamethasone reduces endogenous prostaglandin biosynthesis [42,43]. This is significant because in some people (particularly those with POAG), corticosteroid treatment such as with dexamethasone results in elevated IOP. In addition, inhibition of endogenous PG synthesis by indomethacin treatment, blocks the IOP-reduction activity produced by bunazosin (α₁-adrenergic antagonist) [44], and epinephrine in vitro and in vivo [45,46]; suggesting that endogenous PGs in the conventional pathway play critical role in mediating the reduction of IOP produced by some current glaucoma therapeutics. Since endogenous PGE₂ and PGF_{2α} are the major cyclooxygenase (COX) products of TM cells [47], the role of PGE₂ and PGF_{2α} (and their receptors) in outflow homeostasis will be the emphasis of this section.

Prostaglandins and Outflow Facility

Prostaglandin-induced IOP reduction has been reported by different groups in different species, such as cat, rabbit and monkeys [48–53]. However, there are enormous species differences. The most striking being the lack of effect of FP receptor agonists on rabbit and cat intraocular pressure, despite PGF_{2α} being an efficacious ocular hypotensive in this species [54]. Predictably, non-human primates are a more representative species of man with respect to ocular pharmacology.

Endogenous PGs interact with a multiplicity of receptors to produce their effects. PGD₂ preferentially interacts with two distinct subtypes DP₁ and DP₂. PGE₂ interacts with four distinct receptor subtypes designated EP₁₋₄. PGF_{2α}, prostacyclin, and thromboxane A₂ preferentially interact with only one dedicated subtype, respectively designated FP, IP, and TP. In addition, numerous mRNA splicing variants expand the repertoire of receptors with which PGs and prostamides may interact. Despite the multiplicity of receptors and different intracellular second messenger pathways, topical application of butaprost and AH 13205 (EP₂ selective agonists), latanoprost (FP selective agonist prodrug), bimatoprost (prostamide) and sulprostone (EP₁/EP₃ agonist) produced similar morphologic changes in the aqueous outflow pathways, including trabecular outflow tissue in monkeys [55,56]. After once-daily treatment for a year, the following morphological changes were observed and quantified where possible: In the anterior third of the ciliary body optically empty spaces between the muscle bundles were enlarged. Ultrastructurally these spaces were partially lined with endothelial-like cells. There appeared to be an organized remodeling of the ciliary body with the creation of new drainage channels [55,56]. Changes in the conventional outflow route were also observed for all drugs tested [55]. The loss of ECM from cribriform region of the TM and disconnection of cells should, perhaps, lead to an increase in conventional outflow. Since aqueous humor dynamics were not examined in monkey eyes continuously treated for one year, long-term effects on conventional outflow remains unknown. Certainly, there is no increase in outflow facility after 5 days of treatment with these drugs [38,56] and it appears that effects on intraocular pressure can be entirely attributed to effects on uveoscleral outflow. Recently, in contrast to all PGs and prostamides studied, a selective EP₄ agonist, 3,7-dithia PGE₁, was suggested to increase outflow facility significantly after topical application in monkeys[57], and not affect uveoscleral outflow or aqueous inflow. Such a drug that directly targets conventional outflow tissues represents a novel area for therapeutics. Though the pharmacological mechanisms for the regulation of outflow facility through trabecular meshwork are not clear, prostaglandins represent a class of agents that produce potent and efficacious ocular hypotensive activities. Further studies using receptor-specific agonists/antagonists and different experimental models are needed for more definitive mechanistic information.

Prostaglandin E₂

Among PGs, PGE₂ is the most widely produced in the body and exhibits the most versatile actions. There are four subtypes of EP receptors (EP₁₋₄) mediating PGE₂ actions. All prostanoid receptors are expressed at different levels in human TM tissues. [58–60]. EP₂ receptors are the most abundant isotype of EP receptors in the human TM, while EP₁, EP₄ and EP₃ are expressed at lower levels.

These EP receptors are typical G-protein coupled receptors, each encoded by distinct genes and evoking cellular response via distinct signaling cascades. EP₁ couples with G_q-phospholipase-C-Inositol triphosphate and the protein kinase-C (PKC) pathway. The subtypes EP₂ and EP₄, are both coupled to G_{as}, and signal through stimulation of adenylyl cyclase. EP₃ is G_i-coupled and reduces cAMP [61]. EP₂ and EP₄, though both G_{as}-coupled, are structurally different, and could be involved in different second messenger events after stimulation. Fujino et al. [62] showed that the PGE₂ stimulated Tcf/Lef signaling pathway through EP₂ and EP₄ receptors were involved in different mechanisms. EP₂ was also suggested to couple to G_q, signaling through a PKC-dependent pathway [63]; indicating EP receptors may couple to more than one G protein and signal transduction pathway, instead of coupling exclusively to the classical pathways described above.

Although PGs effectively reduce IOP in monkeys, whether PGs increase outflow facility through the conventional outflow pathway in humans remains unclear for the majority of drugs.

The anterior segment perfusion system is an excellent model for studying TM functional activity, because it isolates the intact TM drainage tissue, maintaining critical association of TM cells with adjacent ECM. Thus, the cellular responses are more representative of those that occur in vivo. Previous studies using this model showed that PGE₁ increased outflow facility through EP₂ and/or EP₄ receptor subtypes, coupled with G_{as} protein activation and intracellular cAMP accumulation [64]. In addition, 24h of high pressure (30 mmHg) in the anterior segment perfusion system decreased EP₂ mRNA level in trabecular meshwork [65]. These data suggest that the endogenous EP system in the conventional outflow pathway plays a role in monitoring and regulating IOP. However, the receptor mediated cellular and molecular mechanisms underlying the effect on conventional outflow pathway remains to be elucidated.

Based on current literature, it is believed that TM drainage tissue is highly functional, responding to various stimuli. For example, substances that relax TM tissue or increase ECM turnover lead to less resistance to aqueous outflow and hence enhance outflow facility [66]. It was shown that a selective EP₂ agonist elicited relaxation of carbachol-precontracted TM strips and ciliary muscle [66,67]. Activation of maxi-K channel-induced hyperpolarization was suggested to be involved in this effect [67]. Conversely, the non-selective PG, PGE₂ was reported to elicit contraction in the iris sphincter model of bovine and cat through phosphorylation of myosin light chain (MLC) and p42/p44 mitogen-activated protein (MAP) kinase [68]. Its effects in human are unknown, but contraction of the ciliary muscle by PGE₂ would facilitate outflow through physically opening the TM outflow pathway. The diverse effects of PGE₂ on different tissues reflect the importance of receptor expression profiles for each tissue. Taken together, these results suggest the involvement of multiple receptor subtypes in conventional outflow regulation.

It was reported that Cyr61 mRNA (factor involved in tissue remodeling) expression was up-regulated through EP₂ receptor stimulation in the isolated cat iris [69] and human ciliary smooth muscle cells and that a non-selective EP receptors agonist induced proMMP-1 and -3 secretion in ciliary muscle cells [70]. Currently unknown is whether EP₂ specifically mediates the secretion of MMPs and ECM turnover in TM cells. There is increasing evidence suggest that the resistance to aqueous humor drainage through TM drainage tissue is in part dependent on the composition of ECM in this tissue [21,23]. For example, outflow facility increases upon exogenous introduction of MMPs into conventional tract [23] or by altering endogenously produced MMPs by conventional outflow cells [71,72]. Thus, further investigation into EP mediated effects on MMPs in the conventional outflow pathway is necessary.

PGF_{2α} and its Analogues

PGF_{2α} and FP receptor agonists are agents that effectively reduce IOP and their effects on ocular physiology have been investigated extensively in the past few years. FP receptors are G-protein coupled receptors, coupled to G_{αq} and in general, upon activation, trigger phosphoinositide turnover, PKC activation and intracellular calcium release. While FP receptors are expressed in the human TM [73,74], the contribution of PGF_{2α}-mediated effects on conventional outflow, as a portion of effects on total outflow, is uncertain.

The primary mechanism proposed for PGF_{2α} and PGF_{2α}-related compounds on IOP is by increasing MMP secretion, and subsequent increase in ECM turnover and tissue remodeling in the ciliary muscle. PGF_{2α} and PGF_{2α}-related compounds were shown to have direct effects on the expression of MMPs and tissue inhibitors of MMPs in various types of ocular tissue/cells, such as TM, ciliary smooth muscle, iris root and sclera [70,75–79]. While TM tissues appeared altered after PGF_{2α} treatment, until recently it was unclear if outflow facility is increased. A recent study reported that latanoprost increased outflow facility in the human anterior segment perfusion model by 65% within 24 hours [35]. Hydraulic conductivity of the sclera was increased in these studies, similar to that found by others [78,80], but not enough

to account for the magnitude of facility change. The downstream signaling pathways stimulated by $\text{PGF}_{2\alpha}$ that lead to the secretion of MMPs are complicated. For example, PKC, MAP kinase and the small G protein, Rho were suggested to be involved in the $\text{PGF}_{2\alpha}$ -induced expression of Cyr61 and CTGF. P38 and P42/44 MAP kinase pathways were stimulated by $\text{PGF}_{2\alpha}$ analogues in cat iris, human ciliary muscle cells, and non-pigmented epithelial cells that may result in an increase in the secretion and activation of MMPs [81–83]. In addition, it was suggested that the prior up-regulation of COX-2 was involved in latanoprost-stimulated expression of MMP-1 [81].

In addition to effects on ECM turnover, PGs may impact outflow facility by altering the contractility of TM tissue [66]. Having smooth muscle-like properties, the TM contracts when exposed to a thromboxane-mimetic, endothelin or cholinergic agents [84]. $\text{PGF}_{2\alpha}$ and its analogues (travoprost and unoprostone) inhibited endothelin-evoked contraction in bovine TM by inhibition of endothelin-associated transient increases in intracellular Ca^{2+} release, whereas carbachol-induced contraction and baseline tension were not affected, indicating an endothelin-dependent IOP-reduction mechanism [74]. Unoprostone was also suggested to reduce the activity of L-type Ca^{2+} channels and activate maxi- K^{+} channels to relax TM tissue [85,86]. Moreover, $\text{PGF}_{2\alpha}$ and latanoprost stimulated PI turnover, p42/p44 MAPK activation, MLC phosphorylation, and contraction of iridial smooth muscle. An increased level of MLC phosphorylation at a constant intracellular Ca^{2+} level (Ca^{2+} sensitization) in the pig iris muscle is mediated through Rho kinase, but not PKC or MAP kinase. These results show that $\text{PGF}_{2\alpha}$ and its analogue induce TM relaxation through the closure of L-type Ca^{2+} channels, stimulation of maxi- K^{+} channels and inhibition of the rise in intracellular calcium due to other mechanisms. On the other hand, FP receptor stimulation activates Rho kinase and MLC phosphorylation to induce tissue contraction. Currently, it is not entirely clear whether outflow facility is influenced by the effects of PGs on tissue contractility, although recent data in human anterior segments is suggestive [35]. Clearly we need a better understanding of how endogenous PGs influence aqueous humor dynamics.

Prostamides

COX-2 not only converts arachidonic acid to prostanoids, but also catalyzes the conversion of the endocannabinoids anandamide (arachidonic ethanolamide, AEA) and 2-arachidonyl glycerol (2-AG) to prostaglandin ethanolamides (prostamides) and prostaglandin glycerols. Prostamide $\text{F}_{2\alpha}$ and its analogue bimatoprost are by far the most extensively studied. Bimatoprost activates signaling pathways in different cells from $\text{PGF}_{2\alpha}$ in the same cat iris sphincter preparation by mechanisms unrelated to conversion to prostaglandins, activation of prostanoid receptors, enhancement of anandamide levels or gating of TRPV1 vanilloid receptors [87,88]. Bimatoprost also potently contracts tissues, such as uterus [89] and stimulates Cyr 61 expression in ciliary muscle cells, with mechanisms that are distinct from that of $\text{PGF}_{2\alpha}$ [69]. Additionally, prostamide $\text{F}_{2\alpha}$ and its analogue bimatoprost exhibit activities at recombinant FP and native FP receptors, including those in TM cells, with a concentration 1000 times higher than $\text{PGF}_{2\alpha}$ and its derivatives. Due to such distinguishing characteristics from $\text{PGF}_{2\alpha}$, prostamides have been proposed as a group of novel pharmacological agents; an idea that is controversial.

Bimatoprost is an efficient anti-glaucoma agent. It potently lowers intraocular pressure in dogs, primates and humans. Interestingly, clinical studies show that bimatoprost decreases IOP in patients resistant to latanoprost treatment [90]. Despite such data, the impact of prostamides on outflow facility in human is not understood at the physiological level. Prostamides have been shown to elicit several biological responses in TM cells. Prostamide $\text{F}_{2\alpha}$ has been reported to modulate gene expression in both human ciliary muscle and human TM cells [91]. Moreover, bimatoprost stimulated PI turnover and intracellular calcium mobilization in human TM cells

at a concentration that much higher than its acidic analogs [92]. Very recently, we reported that bimatoprost elevated hydraulic conductivity through TM monolayers and significantly increased outflow facility within 48 hours of treatment on human anterior segment perfusion system [34]. These results provide direct evidence that bimatoprost targets the TM and exerts functional effects on conventional outflow.

It has been suggested that prostamides might act as prostaglandins pro-drugs that are bio-activated by simple hydrolysis, because the FP receptor agonists also elicit cellular response in TM cells [91,92]. Contrary to this idea, metabolism studies of bimatoprost in ocular tissue showed that although prostaglandins acids were rapidly hydrolyzed in ocular tissue, such as latanoprost, bimatoprost displayed a very slow rate of 1% or less over a 3-hour incubation time in ocular tissues [93,94]. Given that bimatoprost reduces IOP within 2 hours after a single dose [95], effects on IOP are likely direct. Interpretation of these data remains controversial. More studies are needed to distinguish the action of the prostamides from their corresponding prostaglandins.

Studies evaluating the molecular targets of prostamides were mainly based on agonist-stimulated responses. The recent availability of prostamide-specific antagonists has enabled further characterization of prostamide pharmacology and functional activity [34,96,97]. AGN 211334, a second generation prostamide antagonist, specifically blocks the effects of prostamide $F_{2\alpha}$ and bimatoprost but not $PGF_{2\alpha}$ and FP receptor agonists in the feline iris [34, 96]. These data suggest the existence of a molecular target that specifically recognizes prostamides. We recently reported that AGN 211334 pre-incubation significantly blunted bimatoprost-induced outflow facility enhancement in the human anterior segment perfusion model and hydraulic conductivity through TM monolayers, suggesting a TM target distinct from FP receptors [34]. Interestingly, AGN 211334 alone decreased fluid flux across TM tissue and cell monolayers, providing evidence of a functional endogenous prostamide system in the conventional outflow pathway; an idea that needs to be developed further.

Endocannabinoids

In 1971, Helper and Frank reported that marijuana smoking reduced IOP in human volunteers, with an apparent dose-response relationship. However, the mechanisms of IOP-lowering effects, including cannabinoid activity in conventional outflow pathway are unknown.

Since this original observation, an endogenous cannabinoid system of ligands and receptors has been characterized to explain effects of exogenous compounds like Δ_9 -THC, the psychoactive and IOP-lowering constituent in marijuana. Information about the endogenous cannabinoid system emerged when the first cannabinoid receptor was identified in 1992 [98]. All together, there are two primary cannabinoid receptor isoforms, CB_1 and CB_2 . In general, CB_1 receptors are predominantly found in neurons and CB_2 receptors are localized to immune cells and cells in present peripheral tissues. In general, CB_1 and CB_2 receptors couple by means of $G_{ai/o}$ proteins [99]. The signaling pathway downstream of CB receptors includes adenylyl cyclase [100], MAPK [101] and, in the case of CB_1 , depending on the cell type, ion channels [102]. Interestingly, under specific conditions CB_1 receptors preferentially couple to G_{as} in some cells, including TM cells [103].

There are at least three endogenous ligands that bind to and activate cannabinoid receptors, anandamide, 2-AG and noladin ether (NE). These endocannabinoids are synthesized and released following physiological or pathological stimuli. AEA is synthesized via a phospholipid-dependent pathway comprising a Ca^{2+} -dependent enzyme [104]. AEA acts only as a partial CB_1 agonist, as a weak CB_2 agonist and also activates vanilloid type 1 receptor (TRPV1). While 2-AG activates both CB_1 and CB_2 receptors as a full agonist. In contrast, NE is selective for the CB_1 receptor. Lately, highly selective agonists and antagonists for subtypes

of CB receptors have been synthesized, enabling further investigation of the role of CB receptors in IOP-regulation.

Endocannabinoids in the Conventional Outflow Pathway

The distribution of CB₁ receptors in the conventional outflow tract, including expression by TM and SC cells, was observed both at mRNA and protein levels in human and other species [103,105]. Recently, functional CB₂ receptors were demonstrated in porcine TM cells in culture [106]. Interestingly, the TM is one of few cell types that express both receptor subtypes. Other cell types include rat heart and human colon [107,108].

The first endocannabinoid ligand detected in human, porcine and bovine ocular tissues, including the trabecular meshwork, was anandamide [103,109–111]. Shortly afterwards, anandamide's synthase and hydrolase were also observed in ocular tissues [110]. Additionally, NE and 2-AG were found, with NE discovered to be enzymatically more stable than AEA and 2-AG in ocular tissues [109]. The levels of 2-AG, AEA and palmitoylethanolamide (PEA), in human ocular tissue (cornea, iris, ciliary body, retina, and choroid) from normal and glaucomatous donors were compared. Levels of 2-AG and PEA were significantly reduced in the ciliary body from glaucomatous eyes. PEA is co-synthesized with AEA by most cell types and was proposed to enhance AEA effects mediated by both CB₁ and TRPV₁ receptors [112]. Unfortunately, levels of these endocannabinoids were not examined in the trabecular meshwork, the diseased tissue in glaucoma.

Since the original report by Heppler and Frank in human volunteers, several subsequent studies have found that in addition to cannabis, endogenous and synthetic cannabinoids are also effective IOP-reduction agents in mammals. For example, both anandamide and the synthetic cannabinoid, WIN55212-2, significantly decrease IOP in rabbits, non-human primates, and humans after topical application [113–117]. Conversely, it was found that blockage of endogenous cannabinoid tone by application of cannabinoid receptor (CB₁) antagonist elevated IOP; suggesting the existence of endogenous cannabinoid tone that functions in IOP regulation.

In order to explore whether cannabinoids lower IOP via activity in the conventional outflow tract, selective agonists and antagonists for cannabinoid receptor subtypes were tested using the porcine anterior segment model (which physiologically isolates the conventional tract) and cultured TM cells [106,118–120]. Results show that both CB₁ and CB₂ selective agonists, NE and JWH015 respectively, increased outflow facility in the porcine model. The p42/44 MAP kinase signaling pathway appears involved in outflow facility-elevation [106,119]. Moreover, activation of both cannabinoid receptor subtypes had effects on TM cell migration, morphology, actin cytoskeletal architecture and focal adhesion formation in cultured TM cells [118,120]. CB₂ mediated changes in TM cell contractility appear the result of Rac1-GTPase inhibition and the induction of dephosphorylation of the downstream mediator, cofilin [120]. Similar effects on TM contractility following CB₁ activation were found in another model system. Using strips of bovine TM in an organ bath preparation, cannabinoids were shown to relax the TM in a dose dependent manner through CB₁ receptor activation, and downstream effects on maxi-K⁺ channel mediated changes in membrane potential [67].

The effects of cannabinoids on secretion and activation of MMPs in TM tissues are not yet clear. Only one recent study showed that the secretion and activation of MMP-2 was not significantly changed with CB₁-agonist treatment using the porcine anterior segment perfusion model [119]. However, R(+)-MA, a stable analogue of AEA, stimulated the expression of MMP (-1, -3, -9) and TIMP-1 in human ciliary nonpigmented epithelial (NPE) cells through the increase in expression of COX-2 and COX-2-dependent endogenous PGs. Interestingly, MMP-2 was not changed in this study [121]. Preincubation with CB₁-(AM-251), CB₂-(AM-630), and TRPV₁- (capsazepine) antagonist or the G_{ai/o} protein inactivator, pertussis

toxin, did not suppress *R*(+)-MA-induced COX-2 expression in this study. Given that ECM turnover has been shown to be an important contributor to outflow facility regulation, the cannabinoid effects on ECM turnover needs further investigation.

As with other bioactive endogenous lipid mediators, endocannabinoids are generated on demand, rapidly removed from their molecular targets and degraded. For instance, extracellular AEA appears to be taken up by several cell types via a facilitated transport mechanism [104]. Significantly, an inhibitor of AEA reuptake, AM404, reduces IOP in rabbits [122]. However, AM 404 can also inhibit fatty acid amide hydrolase (FAAH) and activate vanilloid TRPV 1 receptors, making it difficult to determine its precise mechanism of action on IOP. More studies are needed to determine effectiveness of endocannabinoid reuptake blockers as a strategy for IOP control. Considering results with cannabinoid agonists, antagonists and reuptake inhibitors, clearly the endocannabinoid system is involved in homeostasis in the conventional outflow pathway and IOP regulation; unknown is the specific contribution of the conventional outflow pathway.

Lysophospholipids

Lysophospholipids are membrane phospholipid metabolites. Recently, their role as autocrine/paracrine signaling molecules has been recognized to influence a broad range of cellular functions, such as cardiac development, immunity, platelet aggregation, cell movement and vascular permeability [123]. The two best characterized lysophospholipids are sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA). S1P and LPA activity are mediated through G-protein coupled receptors specific for each molecule. LPA binds to four receptor subtypes (LPA 1–4 formerly Edg 2, 4, 7, p24a/GPR23), whereas S1P binds specifically to a group of five receptor subtypes (S1P receptors 1–5, formerly known as Edg1, 3, 5, 6, 8). Not surprisingly, LPA and S1P receptor subtypes are differentially expressed in tissues, likely in alignment with specific functional requirements of each tissue. For example, the dominant receptors expressed by vascular endothelial cells are S1P₁ and S1P₃ receptors and the effects of LPA are generally more restricted to certain types of endothelial cells that express the LPA1 receptor [124].

S1P and the Conventional Outflow Pathway

Potentially significant for intraocular pressure, both S1P and LPA are present in aqueous humor, suggesting a role in aqueous humor dynamics. LPA and its homologues are present in the aqueous humor and the lacrimal gland fluid of the rabbit eye, establishing that they are generated endogenously and appearing to be linked to the physiological control of aqueous humor dynamics [125]. In fact, activation of S1P or LPA receptors in the conventional outflow tract dramatically decreased outflow facility in both porcine and human eyes. For instance, in response to 5 μ M S1P outflow facility decreased by 31% in perfused porcine eyes and by 37% in perfused human eyes after 5 hours of treatment [126,127]. Interestingly, while there were no observable histological changes in the JCT region of the trabecular meshwork of porcine eyes, there was a dramatic increase in the density of giant vacuole structures in the endothelial lining of the aqueous plexus [126]. Such a phenotype suggests that S1P increased strength of cell-cell junction adhesions between endothelial cells in the aqueous plexus. This finding is consistent with known effects of S1P on cell-cell junction assembly in endothelial cells via downstream effects on the small GTPase, Rac 1 and subsequently decreased paracellular permeability [128–130]. Similar to vascular endothelial cells, S1P₁ and S1P₃ receptor subtypes are the dominant receptors expressed by both TM and SC cells of the conventional outflow pathway [126,127]. While S1P had dramatic effects on the endothelial cells of the aqueous plexus in porcine eyes, like changes in inner wall morphology was not observed in human eyes. Instead, moderate increases in circumferential actin architecture were observed in the inner wall cells of S1P-treated eyes [127] and comparable to effects of S1P on circumferential actin assembly in vascular endothelial cells [130].

In addition to potential effects on the inner wall, S1P may impact contractility of the TM, a known contributor to outflow resistance. For example, compounds that decrease contractility of the TM such as actin disruptors, decrease outflow facility [131,132]. Because S1P decreases outflow facility, we expect an S1P-mediated increase in contractility of TM. Indeed, when TM cell were treated with S1P in culture, contractility increased [126]. S1P promoted the phosphorylation of MLC, and the formation of stress fibers and focal adhesion, which were primarily mediated through Rho GTPase activation. Additionally, PKC inhibitor pretreatment partially blocked the MLC phosphorylation stimulated by S1P. Such effects of S1P on TM cell contractility in vitro strongly resemble LPA-mediated changes in MLC phosphorylation, stress fiber and focal adhesion enhancement in vitro and corresponding depression of outflow facility in perfused porcine eyes in situ [126]. The physiological reason for the redundant activities of these two lysophospholipids are unknown, but may be related to the importance of controlling TM cell contractility and/or SC cell adhesion in the conventional tract.

Because two S1P receptor subtypes are expressed by the two different cell types that populate the conventional outflow pathway, the specific mechanisms that underlie S1P-mediated decreases in outflow facility and contributions of each cell type are unknown. S1P activates multiple downstream mediators and produces different functional effects depending on the receptor subtype expressed, the downstream coupled G protein and the type of the cell/tissue. Future studies need to dissect out the relative contribution of S1P receptors (S1P₁ versus S1P₃) on TM versus SC cells toward outflow facility regulation. Additionally, the impact of S1P and/or LPA receptor-specific antagonists on outflow facility should indicate the level, location and specific receptors in the conventional outflow pathway that participate in endogenous lysophospholipid signaling to regulate outflow facility.

Expert commentary

Overview

Based upon consistent data from a number of large clinical trials over the past several years, glaucoma management today and for the foreseeable future will involve treatments that lower IOP. Fortunately, significant progress has been made in the medical management of glaucoma. The introduction of prostaglandin analogues has provided a more efficacious and better tolerated class of drugs that only need to be taken once a day, instead twice or more with previous medications. Significantly, prostaglandin analogues are the first chronically prescribed glaucoma medication that increases aqueous humor flow out of the eye. The effectiveness of prostaglandin analogues at increasing uveoscleral outflow, the secondary route for outflow, emphasizes the tremendous potential for the future introduction of a new class of drugs that increase conventional outflow, the primary and pressure-dependent route for outflow.

Unfortunately, progress toward the development of drugs that directly target the conventional outflow pathway has been hampered by a lack of understanding of conventional outflow physiology. Specifically, mechanisms that regulate the resistance to aqueous humor flow through the conventional tract and out of the eye are currently not fully characterized. Slowly, we are beginning to uncover the identity and role of structural elements, adhesion proteins, extracellular matrix components and signaling molecules that participate in the complicated process of outflow resistance regulation. All of these constituents represent viable candidates for anti-glaucoma drug development. In this review, we described the presence and participation of endogenous extracellular lipids in paracrine signaling within conventional tissues, their role in conventional outflow regulation and their respective receptors as candidates for glaucoma drug development. In particular, we reviewed the effects of prostaglandins, endocannabinoids and lysophospholipids on conventional outflow function.

Five-year view

Our current knowledge of endogenous lipid involvement in paracrine signaling in the conventional outflow pathway is rather limited. In the next five years, we not only expect to know more about the specific lipid mediators that participate in conventional outflow regulation, but also have a better understanding of the complexity of interaction among these lipid mediators. Such knowledge is essential for a rational strategy to maximally modify homeostasis, and increase outflow facility.

Due to significant effects on conventional outflow facility, it appears that lipid-based signaling molecules are viable candidates for glaucoma drug development. Moreover, the lipid molecules described in this review all impact mechanisms thought to contribute to conventional outflow resistance. For example, prostaglandins alter secretion of MMPs by trabecular meshwork cells. Alternatively, endocannabinoids and lysophospholipids impact cell contractility, while lysophospholipids also impact cell-cell junction assembly. To complicate these relationships, it appears that manipulations that impact cellular components that mediate contractility influence MMP activation in different cell types, including TM cells [133]. Unknown to a large extent, however, is the relative role of receptor subtypes and their location in the conventional tract, the degree of cross-talk between mediators and the relative contribution of each potential mechanism to total outflow resistance. These unknowns are wide-open areas of research for those interested in understanding the physiology of conventional outflow, the pathology of ocular hypertension in glaucoma and developing a drug that targets the conventional outflow pathway.

A prominent future objective is to characterize the location and expression level of receptor subtypes that bind lipid mediators in the conventional outflow tract. So far, all prostanoid receptor-encoding transcripts have been detected at different levels in human TM tissue [59, 73]. With respect to their protein expression, immunohistochemistry showed that all EP receptor subtypes are expressed by human TM cells [60]. It appears that of the EP receptors in the human TM, EP₂ receptors are the most abundantly expressed isotype [58]. Functional FP receptors were shown in cultures of human TM cells and FP receptor protein in the TM of human eyes [73]. Currently unknown is the expression profile of prostaglandin receptors in SC endothelial cells and the molecular basis for differences in PG versus prostamide pharmacology in ocular cells. Cannabinoid CB₁ receptors were detected in human TM and SC tissue using polyclonal antibodies to the CB₁-receptor [103,105]. CB₂ receptors were detected in porcine TM cells by immunofluorescence microscopy and Western blot studies [106], however their expression in the human conventional tract and specifically in SC is unclear. Moreover, the distribution of the novel cannabinoid receptor, GPR55 has not been examined in ocular tissues yet. With respect to lysophospholipid expression, S1P₁ and S1P₃ receptor transcripts and protein were detected in both human TM and SC cells in culture and in cell lysates prepared from human conventional outflow tissues [126]. LPA₁ receptor transcripts were detected in cDNAs made from mRNA purified from both human TM cell and tissues, however LPA₂ was not present and LPA₃₋₄ receptor expression was not tested [126].

Once the relative receptor subtype expression in outflow cells (e.g.: TM versus SC) is known, the use of subtype specific antagonists will enable determination of the site of action and mechanism responsible for drug effects on conventional outflow. Unfortunately, progress toward understanding the role of specific receptor subtypes on complex physiological processes, such as outflow resistance and IOP regulation have been delayed in some instances by the absence of receptor subtype-specific antagonists. For example, antagonists specific for S1P, LPA and several prostaglandin receptor subtypes are not commercially available. In contrast, antagonists specific for cannabinoid receptors have aided in determining receptor subtype involvement in conventional outflow and IOP regulation.

A major hurdle for the next five years will be to characterize the cross-talk that occurs between paracrine mediators in the conventional outflow pathway. Again, the availability of specific antagonists for receptor subtypes will assist in dissecting relationships. Based upon results in other systems, we currently know that cross-talk occurs between lipid mediators. For example, S1P has been shown to stimulate COX-2 expression in vascular smooth muscle cells, which controls the generation of prostaglandins [134]. Moreover, recent studies report that S1P binds to CB1 cannabinoid receptors, acting as a competitive antagonist [135]. Since anandamide and S1P produce the opposite effects on actin organization and outflow facility, unknown is whether S1P reduces outflow facility via stimulating S1P receptors or via antagonizing CB1 receptors. Finally, in clinical studies administration of NSAIDs partly blocked latanoprost mediated IOP reduction in healthy volunteer as well as ocular hypertension patients [136]. The results imply that FP receptor activation affects COX-2 mediated generation of endogenous prostanoids and prostamides that appear involved in long term IOP reduction. Unknown, however, are the identity of the endogenous prostaglandins that facilitate the IOP lowering effects of latanoprost.

In the vast majority of people, IOP is constrained within a narrow range for a lifetime. Hence, there are likely many processes in place that assure that resistance to conventional outflow, the site of IOP regulation, is tightly controlled. To facilitate such regulation, communication in a paracrine fashion between cells in the outflow pathway appears probable. Here we review several of the molecules expected to participate in such paracrine communication between cells in the conventional tract. Based upon their effects on conventional outflow facility, prostaglandins, lysophospholipids and endocannabinoids likely function as signaling molecules to maintain IOP under control; and their receptors represent exciting candidates for the ultimate development of therapeutics that target the conventional outflow pathway for the treatment of ocular hypertension and glaucoma.

Key Issues

- Glaucoma is one of the leading causes of irreversible blindness in western countries and elevated intraocular pressure is the primary risk factor.
- Elevated intraocular pressure in people with glaucoma results from an increased resistance to aqueous humor outflow through the conventional drainage pathway.
- Even though effective lowering of intraocular pressure in those with glaucoma slows disease progression, current therapeutics do not target the diseased tissue, the conventional outflow pathway.
- Receptors for endogenous bioactive lipid mediators, such as prostaglandins, endocannabinoids and lysophospholipids are expressed by cells that populate the conventional outflow pathway.
- Exogenous application of bioactive lipids impacts the regulation of outflow facility, implicating the involvement of paracrine signaling in the homeostasis of the conventional outflow tissues.
- Receptors and signaling pathways activated by bioactive lipids in the conventional outflow pathway represent viable targets for the development of the next generation of anti-glaucoma medications.

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