Published in final edited form as:

Exp Eye Res. 2018 May ; 170: 20–28. doi:10.1016/j.exer.2018.02.009.

Effect of brimonidine, an α_2 adrenergic agonist, on human meibomian gland epithelial cells

Xi Han, MD, PhD^{a,b}, Yang Liu, MD^a, Wendy R. Kam, MS^a, and David A. Sullivan, MS, PhD^a

^aSchepens Eye Research Institute, Massachusetts Eye and Ear, Harvard Medical School, Boston, MA, USA

^bDepartment of Ophthalmology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Abstract

We recently discovered that the anti-glaucoma pharmaceuticals timolol, a β adrenergic antagonist, and pilocarpine, a cholinergic compound, negatively influence the morphology, proliferative capacity and survival of human meibomian gland epithelial cells (HMGEs). We hypothesize that another class of anti-glaucoma drugs, the α_2 adrenergic agonists, also acts directly on HMGEs to affect their structure and function. We tested this hypothesis. Immortalized (i) HMGEs were cultured with brimonidine, as well as clonidine (α_2 agonist), phenylephrine (α_1 agonist), RX821002 (inverse α_2 agonist) and MK912 (neutral α_2 agonist) for up to 7 days. Cells were counted with a hemocytometer, and evaluated for morphology, signaling pathway activity, protein biomarker expression, and the accumulation of neutral lipids, phospholipids and lysosomes. Our findings demonstrate that brimonidine treatment induces a dose-dependent decrease in Akt signaling and proliferation of iHMGEs. In contrast, brimonidine also promotes a dose-dependent differentiation of iHMGEs, including an increase in neutral lipid, phospholipid and lysosome levels. These effects were paralleled by an inhibition of p38 signaling, and duplicated by cellular exposure to clonidine, but not phenylephrine. Brimonidine also enhanced the cellular content of sterol regulatory binding protein-1, a master regulator of lipid synthesis. Of particular interest, the putative α_2 antagonists, RX821002 and MK912, did not interfere with brimonidine action, but rather stimulated iHMGE differentiation. Our results support our hypothesis and demonstrate that α_2 adrenergic agonists act directly on iHMGEs. However, these compounds do not elicit an overall negative effect. Rather, the α_2 agonists promote the differentiation of iHMGEs.

Keywords

brimonidine; phospholipidosis; α_2 adrenergic agonist; α_2 adrenergic antagonist; meibomian gland dysfunction; dry eye disease

Correspondence Address: David A. Sullivan, MS, PhD, FARVO, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA, USA 02114, TEL: 617-912-0288, david.sullivan@schepens.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Recently, we discovered that the anti-glaucoma pharmaceuticals timolol, a β -adrenergic antagonist, and pilocarpine, a cholinergic compound, negatively influence the morphology, proliferative capacity and survival of human meibomian gland epithelial cells (HMGEs) (Zhang et al., 2017). These effects are adverse, because HMGEs produce secretions that enhance tear film stability, prevent its evaporation, and promote ocular surface health (Knop et al., 2011). Interference with HMGE function leads to meibomian gland dysfunction (MGD), destabilization of the tear film, and evaporative dry eye disease (DED) (Knop et al., 2011). DED, in turn, is characterized by tear film hyperosmolarity, ocular surface inflammation and damage, and pain (Craig et al., 2017).

It is possible that another class of anti-glaucoma drugs, the α_2 adrenergic agonists, may also act directly on HMGEs to affect their structure and function, and lead to the development of MGD and DED. Our rationale is twofold. First, we have identified the mRNAs for α_2 adrenergic receptors in mouse meibomian glands (Knop et al., 2011); and second, topical application of the α_2 adrenergic agonist brimonidine has been shown to elicit foreign body sensations, visual blurring, ocular discomfort and DED (Fraunfelder et al., 2012; Hartleben et al., 2017; Schuman et al., 1997; Servat and Bernardino, 2011; Whitson et al., 2004, 2006). If α_2 adrenergic agonists do induce MGD, this effect would be analogous to that of other anti-glaucoma drugs (Agnifili et al., 2013; Arita et al., 2012; Batra et al., 2014; Cuniffe et al., 2011; Custer and Kent, 2016; Mocan et al., 2016; Uzunosmanoglu et al., 2016), and help to explain further why topical anti-glaucoma treatment often aggravates DED and other ocular surface diseases (Fraunfelder et al., 2012; Servat and Bernardino, 2011; Uzunosmanoglu et al., 2016; Gomes et al., 2017).

The purpose of this investigation was to test our hypothesis that α_2 adrenergic agonists act directly on HMGEs to influence their structure and function. To represent this class of drugs, we chose brimonidine and focused on its effect on the morphology, survival, proliferation and differentiation of HMGEs. We also examined the chemical specificity of brimonidine's action, and the mechanism(s) underlying this compound's influence on these cells.

2. Materials and Methods

2.1 Drugs

Brimonidine (α_2 agonist), clonidine (α_2 agonist) and phenylephrine (α_1 agonist) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and dissolved in keratinocyte serum-free medium (KSFM; Thermo-Fisher Scientific, Grand Island, NY, USA) to prepare 5 mg/ml stocks. Brimonidine concentrations were chosen based upon the topical clinical dose (i.e. 0.2%, which equals 2 mg/ml), as well as studies investigating the levels (i.e. $\sim 50 \mu\text{g/ml}$) of brimonidine in the conjunctiva after topical administration (Acheampong et al., 2002). The α_2 antagonists RX821002 and MK912 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; Corning Cellgro, Manassas, VA, USA) containing 10% fetal bovine serum (FBS; Thermo-Fisher Scientific, Grand Island, NY, USA) to make 10 mg/ml

and 2 mg/ml stocks, respectively. Forskolin and 3-isobutyl-1-methylxanthine (IBMX, both Sigma-Aldrich) were dissolved in ethanol to form 10 mM and 45 mM stocks, respectively. Stocks were further diluted in culture media to the concentrations described.

2.2 Cell Proliferation Assay

Immortalized HMGEs (iHMGEs), which were developed in our laboratory with retroviral human telomerase reverse transcriptase (Liu et al., 2010), were seeded in 12 well plates (3×10^4 cell / well) in KSFM with or without brimonidine for 7 days. KSFM supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μ g/ml bovine pituitary extract (BPE) (Thermo-Fisher Scientific), which are known to promote proliferation of iHMGEs, was used as a positive control (Zhang et al., 2017). Cellular morphology was recorded and cells were counted with a hemocytometer. Experiments were performed in triplicate and repeated at least 3 times.

2.3 Cellular staining

Cells were treated in DMEM/F12 containing 10% FBS in the presence or absence of HCS LipidTOX™ Red phospholipidosis detection reagent for 5-7 days. Cells were stained for lysosome accumulation using LysoTracker® Red DND-99 or LysoTracker® Blue DND-22 and for neutral lipid with HCS LipidTOX™ Green neutral lipid stain (Zhang et al., 2017). All stains were purchased from Thermo-Fisher Scientific. Cells treated with 10 μ g/ml azithromycin (Santa Cruz Biotechnology, Dallas, TX, USA) were used as a positive control for differentiation (Zhang et al., 2017). Each experiment was performed in duplicate and four random pictures were taken of each sample. Experiments were performed at least twice.

2.4 SDS-PAGE and Western blots

In order to investigate signaling pathways in proliferating cells, iHMGEs were maintained in KSFM supplemented with EGF and BPE for 2 days and starved in KSFM overnight. To identify changes to iHMGEs in differentiating conditions, cells were exposed to DMEM/F12 containing 10% FBS for 6 days. In signal pathway experiments, cells were starved overnight in DMEM/F12 containing 1% FBS and then treated with drugs for 15 minutes. In other experiments, cells were grown in DMEM/F12 containing 10% FBS with brimonidine for 6 days prior to lysis and immunoblot as previously described (Zhang et al., 2017). Primary antibodies were specific for phosphorylated phosphoinositide 3-kinase-protein kinase B (p-Akt, 1:4000, Cell Signaling Technology, Danvers, MA, USA), phosphorylated extracellular signal-regulated kinase (p-ERK, 1:2000, Santa Cruz Biotechnology), ERK2 (1:4000, Santa Cruz Biotechnology), phosphorylated p38 mitogen activated protein kinase (p-p38, 1:1000, Cell Signaling Technology), p38 (1:1000, Cell Signaling Technology), phosphorylated c-Jun N-terminal kinases (p-JNK, 1:2000, Santa Cruz Biotechnology), JNK (1:1000, Santa Cruz Biotechnology), sterol regulatory element-binding protein 1 (SREBP-1, H-160, 1:500, Santa Cruz Biotechnology), light chain 3A (LC3A, 1:1000, Cell Signaling Technology), lysosomal-associated membrane protein 1 (LAMP-1, H4A3-s, 1:500, Developmental Studies Hybridoma Bank, Iowa City, IA) and β -actin (1:10,000, Cell Signaling Technology). Densitometry was performed with publicly available image-processing software (ImageJ; <http://rsb.info.nih.gov/ij>). Experiments were performed in triplicate and repeated at least twice.

2.5 cAMP ELISA

Cells were plated in 96 well plates at a density of 5×10^4 cells / well overnight, then treated with brimonidine, forskolin, or IBMX, alone or in combination, for 15 minutes. Cellular adenosine 3,5-cyclic monophosphate (cAMP) was measured using the Amersham cAMP Biotrak Enzymeimmunoassay (EIA) System (GE Health Care, Buckinghamshire, United Kingdom), according to the nonacetylation protocol supplied by the manufacturer. Forskolin, an activator of adenylate cyclase, and IBMX, a competitive inhibitor of phosphodiesterase, were used as positive controls for cAMP accumulation (Essayn, 2001). A standard curve in duplicate was run in each assay. Three independent experiments in triplicate were conducted.

2.6 Statistics

We performed one-way analysis of variance and unpaired, 2-tailed Student's t-tests. All statistical analyses were performed using commercially available software (Prism 5; GraphPad Software, Inc, La Jolla, CA, USA).

3. Results

3.1 Influence of brimonidine on the morphology, survival and proliferation of iHMGECS

To evaluate the effect of brimonidine on the proliferation of iHMGECS, cells were treated with various doses of this compound (i.e. 500 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$) or vehicle and observed for seven days. These brimonidine concentrations were equivalent to one-fourth the clinical dose of 2 mg/ml, the conjunctival level (i.e. physiological) of $\sim 50 \mu\text{g/ml}$ that follows topical administration (Acheampong et al., 2002), and one-tenth the latter amount. It is likely that HMGECS *in vivo* are exposed to the "physiological" concentration.

As illustrated in Figure 1, exposure to 500 $\mu\text{g/ml}$ brimonidine induced cell rounding and detachment within three days, and a significant reduction in cell number. In contrast, neither the 50 $\mu\text{g/ml}$ nor the 5 $\mu\text{g/ml}$ brimonidine concentrations had any effect on iHMGECS morphology or number (Figure 1 A, B).

Given the impact of high dose brimonidine on iHMGECS survival, we examined whether this compound has a dose-dependent effect on the Akt signaling pathway. Akt promotes cell survival, growth and proliferation (Jin et al., 2012). As demonstrated in Figure 1C, the 500 $\mu\text{g/ml}$ brimonidine treatment, but not the other brimonidine concentrations, significantly decreased p-Akt levels.

3.2 Effects of brimonidine, clonidine and phenylephrine on the differentiation of iHMGECS

To determine whether brimonidine influences the differentiation of iHMGECS, we cultured cells for 5–7 days in DMEM/F12 supplemented with 10% FBS, and vehicle, or 500 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$ of brimonidine, or azithromycin (10 $\mu\text{g/ml}$) as a positive control. We then stained cells for the analysis of lysosomes and neutral lipids. As demonstrated in Figure 2 A, the 500 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ concentrations of brimonidine, but not the 5 $\mu\text{g/ml}$ level, stimulated a pronounced accumulation of lysosomes and neutral lipids in iHMGECS. The

neutral lipids co-localized within lysosomes, in a manner analogous to that following azithromycin treatment (Figure 2A, “Merge”).

To examine the adrenergic specificity of brimonidine’s differentiative influence, we cultured iHMGEs with clonidine, another α_2 adrenergic agonist, and phenylephrine, an α_1 adrenergic agonist, for 7 days. Initially, we chose clonidine concentrations of 500 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ to match those of brimonidine that stimulated cell differentiation. However, the 500 $\mu\text{g/ml}$ clonidine dose was toxic (data not shown), and consequently we reduced the upper clonidine level to 200 $\mu\text{g/ml}$. As illustrated in Figure 2B, the 200 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ clonidine treatments duplicated brimonidine’s effect on the accumulation of lysosomes and neutral lipids. In contrast, iHMGE exposure to 500 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$ of phenylephrine had no impact on these cellular parameters (Figure 2 C).

3.3 Impact of brimonidine, clonidine and phenylephrine on phospholipid accumulation in iHMGEs

We have previously discovered that azithromycin, a potent phospholipidosis (PLD)-inducing cationic amphiphilic drug (CAD) (Ribeiro et al., 2009; Tyteca et al., 2001), elicits a PLD-like effect in iHMGEs (Liu et al., 2014). Thus, azithromycin promotes the accumulation of phospholipids, cholesterol and lysosomal lamellar bodies in iHMGEs (Liu et al., 2014). Given that the intracellular accumulation of phospholipids in lysosomes is one of the hallmark features of PLD (Reasor and Kacew, 2001; Sadrieh, 2010), we sought to determine whether brimonidine and clonidine also induce a PLD-like effect in iHMGEs. For comparison, we tested the influence of phenylephrine as well.

As shown in Figure 3, brimonidine at a concentration of 500 $\mu\text{g/ml}$, and clonidine at 200 $\mu\text{g/ml}$, both stimulated phospholipid accumulation in iHMGE lysosomes. Their PLD-like effect was similar to that of azithromycin (Figure 3). Phenylephrine at 500 $\mu\text{g/ml}$ had no demonstrable influence on phospholipid appearance (Figure 3).

3.4 Effects of brimonidine, clonidine and phenylephrine on signaling pathways in iHMGEs

To determine whether the α_2 adrenergic agonist induction of iHMGE differentiation involves the activity of signaling pathways, we evaluated the ability of 500 $\mu\text{g/ml}$ brimonidine, 200 $\mu\text{g/ml}$ clonidine and 500 $\mu\text{g/ml}$ phenylephrine to modulate the levels of Akt, ERK, p38 and JNK, with or without phosphorylation. These pathways have all been linked to α_2 adrenergic receptors (Caine, 1998; Kajiya et al., 2012; Kobayashi et al., 2007).

Our findings demonstrate that all three adrenergic agonists decreased p-Akt expression, as compared to that of the vehicle control (Figure 4A). Brimonidine and clonidine also reduced p-p38 levels, whereas only phenylephrine suppressed p-ERK (Figure 4A). None of the drugs altered p-JNK.

As part of these analyses we tested whether 500 $\mu\text{g/ml}$ brimonidine exposure, in the presence or absence of forskolin (10^{-6} M) or IBMX (1 mM), influences cAMP levels in iHMGEs. Our data show that both forskolin and IBMX stimulate cAMP accumulation, but that brimonidine had no effect (Figure 4B). Given this observation, we did not examine the

comparative influence of either clonidine or phenylephrine on the adenylate cyclase pathway.

3.5 Influence of brimonidine on the expression of protein biomarkers of differentiation in iHMGEs

We have previously discovered that the differentiation of primary and immortalized HMGEs is associated with an upregulation of genes linked to lysosomes, lipids, phagosomes and autophagy (Sullivan et al., 2014). To assess whether brimonidine's influence on iHMGE differentiation involves the translation of such genes, we treated cells with brimonidine for 6 days and analyzed cell lysates for SREBP-1, LAMP-1 and LC3A. SREBP-1 is a key lipogenesis regulator, which increases the synthesis of enzymes involved in fatty acid, lipid and cholesterol production (Eberle et al., 2004). SREBP-1 is activated by cleavage of a precursor into the mature form (Horton et al., 2002), resulting in a cluster of protein bands between 59 and 68 kDa (Gosmain et al., 2005). LAMP-1 and LC3 are biomarkers for lysosomes and autophagosomes, respectively (Eskelinen, 2006; Tanida et al., 2008).

As shown in Figure 5A, treatment with 500 µg/ml brimonidine increased the mature, and decreased the precursor, forms of SREBP-1, indicating that brimonidine promotes the conversion of SREBP-1 to the active moiety. Brimonidine also enhanced the expression of both LAMP-1 and LC3A (Figure 5B).

3.6 Effect of $\alpha 2$ adrenergic antagonists on the brimonidine-induced differentiation of iHMGEs

To determine whether $\alpha 2$ adrenergic receptor antagonists interfere with brimonidine-induced differentiation of iHMGEs, we cultured cells for 5 days with vehicle, azithromycin (10 µg/ml, positive control), or RX821002 (1,000 µg/ml) or MK-912 (1,000 µg/ml) in the presence or absence of brimonidine (500 µg/ml). RX821002 demonstrates inverse agonist properties at $\alpha 2$ adrenergic receptors (Clarke and Harris, 2002, Murrin et al., 2000), which means that it should induce a pharmacological response opposite to that of brimonidine. MK-912 acts as a neutral agonist (Murrin et al., 2000), and should have no activity in the absence of brimonidine, but be able to inhibit brimonidine action. We selected antagonist concentrations based upon reported binding affinities for $\alpha 2A$ adrenergic receptor subtype (Table 1) (Lomasney et al., 1991, MacDonald et al., 1997, Uhlen et al., 1995).

Our results show that neither RX821002 nor MK-912 acted as overall antagonists of brimonidine-induced differentiation of iHMGEs (Figures 6A, B). Indeed, both compounds stimulated the accumulation of lysosomes and/or neutral lipids. At the RX821002 and MK-912 concentrations used, though, as with brimonidine at 500 µg/ml, there were relatively few cells remaining after 5 days of culture. Consequently, we reduced the concentrations of RX821002 and MK-912 to 100 and 200 µg/ml and examined their effect on iHMGEs after 2 days of culture. As illustrated in Figure 6C, both RX821002 and MK-912 acted like $\alpha 2$ adrenergic receptor agonists and increased the iHMGE content of lysosomes and neutral lipids.

4. Discussion

Our results show that brimonidine exposure induces a dose-dependent reduction in iHMGEK Akt signaling and proliferation. In contrast, brimonidine promotes a dose-dependent differentiation of iHMGEKs, including an increase in neutral lipid, phospholipid and lysosome levels. These effects were associated with an inhibition of p38 signaling, and were reproduced by cellular exposure to clonidine, but not phenylephrine. Brimonidine also enhanced the cellular content of SREBP, LAMP-1 and LCA3. Of particular interest, the putative α_2 antagonists, RX821002 and MK912, did not inhibit brimonidine action, but rather stimulated iHMGEK differentiation. Overall, our findings support our hypothesis and demonstrate that α_2 adrenergic agonists act directly on iHMGEKs. However, unlike other anti-glaucoma pharmaceuticals (e.g. timolol and pilocarpine), these α_2 adrenergic drugs do not cause a general negative effect. Rather, the α_2 agonists stimulate iHMGEK differentiation.

Topical clonidine was the first α_2 adrenergic agonist to be marketed for the treatment of glaucoma, but brimonidine is more widely used because of its higher α_2 adrenergic receptor selectivity and lower systemic side effects (Apatachioae and Chiselita, 1999). The α_1 agonist, phenylephrine, does not have a role in glaucoma therapy. The stimulation of iHMGEK lipid production by brimonidine and clonidine, but not by phenylephrine, suggests that α_2 , and not α_1 , adrenergic receptor agonists alter the activity of one or more signaling pathways that promote differentiation. One such pathway could be that of p38. We found that brimonidine and clonidine, but not phenylephrine, decreased the phosphorylation of p38. This response may facilitate lipid accumulation, because p38 typically suppresses lipid synthesis. Thus, a reduction in p38 activity is paralleled by a rise in liver fat levels (Xiong et al., 2007), as well as an increase in adipogenesis (He et al., 2013). Whether a specific link exists between p38 suppression and lipid accrual in HMGEKs, though, remains to be determined.

We discovered that brimonidine and clonidine stimulate phospholipid accumulation in iHMGEK lysosomes, and that this PLD-like effect is analogous to that caused by azithromycin. This finding suggests that these α_2 adrenergic agonists may be CADs. In support of this hypothesis, we observed the change in phospholipid dynamics by using the LipidTOX Red reagent, which has been utilized extensively to identify CAD-induced PLD (Shahane et al., 2014). In addition, brimonidine, like CADs, is positively charged at physiological pH (Ueda et al., 2000), and could incorporate, like CADs, into the negatively charged lysosomal membranes (Muehlbacher et al., 2012). Further, clonidine has been postulated to be a CAD (Kubo, 2016). If brimonidine and clonidine are CADs, they may act through multiple possible mechanisms to promote PLD. These include stimulating the synthesis, and/or preventing the degradation, of phospholipids (Halliwell 1997, Muehlbacher et al., 2012).

To begin to understand the mechanism of lipid synthesis in iHMGEKs in response to brimonidine treatment, we investigated the expression of three relevant proteins. Brimonidine elicits upregulation of SREBP-1, LAMP-1 and LC3A in iHMGEKs. SREBP-1, a master regulator of lipid synthesis (Eberle et al., 2004), is synthesized as an inactive

precursor and cleaved into an active mature form. Brimonidine triggers the conversion of the SREBP-1 precursor to the mature form, promoting lipid accumulation in iHMGEs. LAMP-1 is a transmembrane protein, which resides primarily across lysosomal membranes (Carlsson and Fukuda, 1989). Brimonidine stimulation results in a dose-dependent upregulation of LAMP-1, which is consistent with increased lysosome staining. Of particular note, brimonidine at high concentration contributes to a prominent increase of LC3A, one isoform of LC3, which is the most widely-monitored autophagy-related protein (Klionsky et al., 2016). At a physiological concentration, brimonidine promotes iHMGE differentiation (more accumulation of neutral lipids and lysosomes) but not autophagy (no upregulation of LC3A). However, at a high concentration, brimonidine induces both terminal differentiation (more striking neutral lipid and lysosomal accumulation) and autophagy (obvious upregulation of LC3A), suggesting a role for autophagy in terminal differentiation in the meibomian gland (Fischer et al., 2017).

To our surprise, the two α_2 adrenergic antagonists, RX821002 and MK912, did not inhibit, but rather duplicated, the differentiative effects of brimonidine and clonidine on iHMGEs. We are unaware of other studies reporting α_2 adrenergic agonist activity for RX821002 and MK912. However, such an action is not unique. The selective estrogen receptor modulators, tamoxifen, raloxifene, and toremifene, all exhibit mixed agonist and antagonist ability, depending on the target tissue (Cosman and Lindsay, 1999; Gallo and Kaufman, 1997). Opioids are also known to act as agonists or antagonists, depending upon the receptor type (Hoskin and Hanks, 1991). It may be that the iHMGE differentiation reflects a cell-specific response to RX821002 and MK912.

Given our discovery that brimonidine stimulates iHMGE differentiation, how then does the topical application of brimonidine elicit foreign body sensations, visual blurring, ocular discomfort and DED (Fraunfelder et al., 2012; Hartleben et al., 2017; Schuman et al., 1997; Servat and Bernardino, 2011; Whitson et al., 2004, 2006)? These adverse events may be related to the corneal toxicity (Robciuc et al., 2017), goblet cell loss (Aydin et al., 2014), and conjunctival hyperaemia and inflammation (Hartleben et al., 2017 and Serle, 1996) associated with brimonidine administration.

Our goal is to determine whether brimonidine is effective *in vivo* by using a mouse model of obstructive MGD. This model, which was developed to facilitate understanding of MGD pathophysiology, is generated by feeding HR-1 hairless mice a special diet with limited lipid content (Miyake et al., 2016). These mice develop markedly plugged (i.e. obstructed) meibomian gland orifices, hyperkeratinization of the terminal duct epithelium and toothpaste-like meibomian gland secretions. Hyperkeratinization of the ductal epithelium and reduced meibum quality, in turn, contribute significantly to the luminal plugging found in human obstructive MGD (Knop et al., 2011). Moreover, topical application of azithromycin, a CAD, ameliorates the MGD in this model (Miyake et al., 2016). Hence, this mouse model, which mimics many of the characteristics of human MGD, may well be responsive to brimonidine therapy.

Overall, unlike other anti-glaucoma drugs that induce MGD (Agnifili et al., 2013; Arita et al., 2012; Batra et al., 2014; Cunniffe et al., 2011; Custer and Kent, 2016; Mocan et al.,

2016; Uzunosmanoglu et al., 2016), our findings suggest that $\alpha 2$ adrenergic agonists, especially at physiological levels, are beneficial for HMGEc function.

Acknowledgments

Funding

This work was supported by NIH grant EY028653 and the Margaret S. Sinon Scholar in Ocular Surface Research Fund.

Abbreviations

AKT	phosphoinositide 3-kinase-protein kinase B
BPE	bovine pituitary extract
CAD	cationic amphiphilic drug
cAMP	adenosine 3,5-cyclic monophosphate
DED	dry eye disease
DMEM/F12	1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
IBMX	3-isobutyl-1-methylxanthine
iHMGEcs	immortalized human meibomian gland epithelial cells
JNK	c-Jun N-terminal kinases
KSFM	keratinocyte serum-free medium
LAMP-1	lysosomal-associated membrane protein 1
LC3A	light chain 3A
MGD	Meibomian gland dysfunction
p38	p38 mitogen activated protein kinase
SREBP-1	sterol regulatory element-binding protein 1

References

- Acheampong AA, Shackleton M, John B, Burke J, Wheeler L, Tang-Liu D. Distribution of brimonidine into anterior and posterior tissues of monkey, rabbit, and rat eyes. *Drug Metab Dispos.* 2002; 30:421–429. [PubMed: 11901096]
- Agnifili L, Fasanella V, Costagliola C, Ciabattini C, Mastropasqua R, Frezzotti P, Mastropasqua L. In vivo confocal microscopy of meibomian glands in glaucoma. *Br J Ophthalmol.* 2013; 97:343–349. [PubMed: 23269683]

- Apatachioae I, Chiselita D. Alpha-2 adrenergic agonists in the treatment of glaucoma. *Oftalmologia*. 1999; 47:35–40. [PubMed: 10641099]
- Arita R, Itoh K, Maeda S, Maeda K, Furuta A, Tomidokoro A, Aihara M, Amano S. Comparison of the long-term effects of various topical antiglaucoma medications on meibomian glands. *Cornea*. 2012; 31:1229–1234. [PubMed: 22406943]
- Aydin Kurna S, Acikgoz S, Altun A, Ozbay N, Sengor T, Olcaysu OO. The effects of topical antiglaucoma drugs as monotherapy on the ocular surface: a prospective study. *J Ophthalmol*. 2014; 2014:460483. [PubMed: 25009742]
- Batra R, Tailor R, Mohamed S. Ocular surface disease exacerbated glaucoma: optimizing the ocular surface improves intraocular pressure control. *J Glaucoma*. 2014; 23:56–60. [PubMed: 22828007]
- Caine M. Alpha-adrenergic mechanisms in dynamics of benign prostatic hypertrophy. *Urology*. 1988; 32:16–20.
- Carlsson SR, Fukuda M. Structure of human lysosomal membrane glycoprotein 1. Assignment of disulfide bonds and visualization of its domain arrangement. *J Biol Chem*. 1989; 264:20526–20531. [PubMed: 2584229]
- Clarke RW, Harris J. RX 821002 as a tool for physiological investigation of alpha(2)-adrenoceptors. *CNS Drug Rev*. 2002; 8:177–192. [PubMed: 12177687]
- Cosman F, Lindsay R. Selective estrogen receptor modulators: clinical spectrum. *Endocr Rev*. 1999; 20:418–434. [PubMed: 10368777]
- Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo CK, Liu Z, Nelson JD, Nichols JJ, Tsubota K, et al. TFOS DEWS II Definition and Classification Report. *Ocul Surf*. 2017; 15:276–283. [PubMed: 28736335]
- Cunniffe MG, Medel-Jimenez R, Gonzalez-Candial M. Topical antiglaucoma treatment with prostaglandin analogues may precipitate meibomian gland disease. *Ophthal Plast Reconstr Surg*. 2011; 27:e128–129.
- Custer PL, Kent TL. Observations on Prostaglandin Orbitopathy. *Ophthal Plast Reconstr Surg*. 2016; 32:102–105.
- Eberle D, Hegarty B, Bossard P, Ferre P, Fougelle F. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie*. 2004; 86:839–848. [PubMed: 15589694]
- Eskelinen EL. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol Aspects Med*. 2006; 27:495–502. [PubMed: 16973206]
- Essayan DM. Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol*. 2001; 108:671–680. [PubMed: 11692087]
- Fischer H, Fumicz J, Rossiter H, Napirei M, Buchberger M, Tschachler E, Eckhart L. Holocrine Secretion of Sebum Is a Unique DNase2-Dependent Mode of Programmed Cell Death. *J Invest Dermatol*. 2017; 137:587–594. [PubMed: 27771328]
- Fraunfelder FT, Sciubba JJ, Mathers WD. The role of medications in causing dry eye. *J Ophthalmol*. 2012; 2012:285851. [PubMed: 23050121]
- Gallo MA, Kaufman D. Antagonistic and agonistic effects of tamoxifen: significance in human cancer. *Semin Oncol*. 1997; 24:S1-71–S71-80.
- Gomes JAP, Azar DT, Baudouin C, Efron N, Hirayama M, Horwath-Winter J, Kim T, Mehta JS, Messmer EM, Pepose JS, et al. TFOS DEWS II iatrogenic report. *Ocul Surf*. 2017; 15:511–538. [PubMed: 28736341]
- Gosmain Y, Dif N, Berbe V, Loizon E, Rieusset J, Vidal H, Lefai E. Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J Lipid Res*. 2005; 46:697–705. [PubMed: 15627654]
- Halliwell WH. Cationic amphiphilic drug-induced phospholipidosis. *Toxicol Pathol*. 1997; 25:53–60. [PubMed: 9061852]
- Hartleben C, Parra JC, Batoosingh A, Bernstein P, Goodkin M. A Masked, Randomized, Phase 3 Comparison of Triple Fixed-Combination Bimatoprost/Brimonidine/Timolol versus Fixed-Combination Brimonidine/Timolol for Lowering Intraocular Pressure. *J Ophthalmol*. 2017; 2017:4586763. [PubMed: 29057117]

- He Z, Zhu HH, Bauler TJ, Wang J, Ciaraldi T, Alderson N, Li S, Raquil MA, Ji K, Wang S, et al. Nonreceptor tyrosine phosphatase Shp2 promotes adipogenesis through inhibition of p38 MAP kinase. *Proc Natl Acad Sci U S A*. 2013; 110:E79–88. [PubMed: 23236157]
- Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002; 109:1125–1131. [PubMed: 11994399]
- Hoskin PJ, Hanks GW. Opioid agonist-antagonist drugs in acute and chronic pain states. *Drugs*. 1991; 41:326–344. [PubMed: 1711441]
- Jin M, Hwang SM, Davies AJ, Shin Y, Bae JS, Lee JH, Lee EB, Song YW, Park K. Autoantibodies in primary Sjogren's syndrome patients induce internalization of muscarinic type 3 receptors. *Biochim Biophys Acta*. 2012; 1822:161–167. [PubMed: 22137887]
- Kajiya M, Ichimonji I, Min C, Zhu T, Jin JO, Yu Q, Almazroo SA, Cha S, Kawai T. Muscarinic type 3 receptor induces cytoprotective signaling in salivary gland cells through epidermal growth factor receptor transactivation. *Mol Pharmacol*. 2012; 82:115–124. [PubMed: 22511543]
- Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. 2016; 12:1–222. [PubMed: 26799652]
- Knop E, Knop N, Millar T, Obata H, Sullivan DA. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. *Invest Ophthalmol Vis Sci*. 2011; 52:1938–1978. [PubMed: 21450915]
- Kobayashi F, Yageta Y, Segawa M, Matsuzawa S. Effects of imidafenacin (KRP-197/ONO-8025), a new anti-cholinergic agent, on muscarinic acetylcholine receptors. High affinities for M3 and M1 receptor subtypes and selectivity for urinary bladder over salivary gland. *Arzneimittelforschung*. 2007; 57:92–100. [PubMed: 17396619]
- Kubo Y, Seko N, Usui T, Akanuma S, Hosoya K. Lysosomal Trapping Is Present in Retinal Capillary Endothelial Cells: Insight into Its Influence on Cationic Drug Transport at the Inner Blood-Retinal Barrier. *Biol Pharm Bull*. 2016; 39:1319–1324. [PubMed: 27476940]
- Liu S, Hatton MP, Khandelwal P, Sullivan DA. Culture, immortalization, and characterization of human meibomian gland epithelial cells. *Invest Ophthalmol Vis Sci*. 2010; 51:3993–4005. [PubMed: 20335607]
- Liu Y, Kam WR, Ding J, Sullivan DA. One man's poison is another man's meat: using azithromycin-induced phospholipidosis to promote ocular surface health. *Toxicology*. 2014; 320:1–5. [PubMed: 24613571]
- Lomasney JW, Cotecchia S, Lorenz W, Leung WY, Schwinn DA, Yang-Feng TL, Brownstein M, Lefkowitz RJ, Caron MG. Molecular cloning and expression of the cDNA for the alpha 1A-adrenergic receptor. The gene for which is located on human chromosome 5. *J Biol Chem*. 1991; 266:6365–6369. [PubMed: 1706716]
- MacDonald E, Kobilka BK, Scheinin M. Gene targeting-homing in on alpha 2-adrenoceptor-subtype function. *Trends Pharmacol Sci*. 1997; 18:211–219. [PubMed: 9227000]
- Miyake H, Oda T, Katsuta O, Seno M, Nakamura M. Meibomian gland dysfunction model in hairless mice fed a special diet with limited lipid content. *Invest Ophthalmol Vis Sci*. 2016; 57:3268–75. [PubMed: 27327582]
- Mocan MC, Uzunosmanoglu E, Kocabeyoglu S, Karakaya J, Irkec M. The Association of Chronic Topical Prostaglandin Analog Use With Meibomian Gland Dysfunction. *J Glaucoma*. 2016; 25:770–774. [PubMed: 27513901]
- Muehlbacher M, Tripal P, Roas F, Kornhuber J. Identification of drugs inducing phospholipidosis by novel in vitro data. *ChemMedChem*. 2012; 7:1925–1934. [PubMed: 22945602]
- Murrin LC, Gerety ME, Happe HK, Bylund DB. Inverse agonism at alpha(2)-adrenoceptors in native tissue. *Eur J Pharmacol*. 2000; 398:185–191. [PubMed: 10854829]
- Reasor MJ, Kacew S. Drug-induced phospholipidosis: are there functional consequences? *Exp Biol Med* (Maywood). 2001; 226:825–830. [PubMed: 11568304]
- Ribeiro CM, Hurd H, Wu Y, Martino ME, Jones L, Brighton B, Boucher RC, O'Neal WK. Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia. *PLoS One*. 2009; 4:e5806. [PubMed: 19503797]

- Robciuc A, Witos J, Ruokonen SK, Rantamaki AH, Pisella PJ, Wiedmer SK, Holopainen JM. Pure Glaucoma Drugs Are Toxic to Immortalized Human Corneal Epithelial Cells, but They Do Not Destabilize Lipid Membranes. *Cornea*. 2017; 36:1249–1255. [PubMed: 28825921]
- Sadrieh, N. The Regulatory Challenges of Drug-induced Phospholipidosis. 2010. Available at: <http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/advisorycommitteeforpharmaceuticalscienceandclinicalpharmacology/ucm210798.pdf>; Accessed December 14, 2016.
- Schuman JS, Horwitz B, Choplin NT, David R, Albracht D, Chen K. A 1-year study of brimonidine twice daily in glaucoma and ocular hypertension. A controlled, randomized, multicenter clinical trial. Chronic Brimonidine Study Group. *Arch Ophthalmol*. 1997; 115:847–852. [PubMed: 9230823]
- Serle JB. A comparison of the safety and efficacy of twice daily brimonidine 0.2% versus betaxolol 0.25% in subjects with elevated intraocular pressure. The Brimonidine Study Group III. *Surv Ophthalmol*. 1996; 41(Suppl 1):S39–47. [PubMed: 8970248]
- Servat JJ, Bernardino CR. Effects of common topical antiglaucoma medications on the ocular surface, eyelids and periorbital tissue. *Drugs Aging*. 2011; 28:267–282. [PubMed: 21428462]
- Shahane SA, Huang R, Gerhold D, Baxa U, Austin CP, Xia M. Detection of phospholipidosis induction: a cell-based assay in high-throughput and high-content format. *J Biomol Screen*. 2014; 19:66–76. [PubMed: 24003057]
- Sullivan DA, Liu Y, Kam WR, Ding J, Green KM, Shaffer SA, Hatton MP, Liu S. Serum-induced differentiation of human meibomian gland epithelial cells. *Invest Ophthalmol Vis Sci*. 2014; 55:3866–3877. [PubMed: 24867579]
- Tanida I, Ueno T, Kominami E. LC3 and Autophagy. *Methods Mol Biol*. 2008; 445:77–88. [PubMed: 18425443]
- Tyteca D, Van Der Smissen P, Van Bambeke F, Leys K, Tulkens PM, Courtroy PJ, Mingeot-Leclercq MP. Azithromycin, a lysosomotropic antibiotic, impairs fluid-phase pinocytosis in cultured fibroblasts. *Eur J Cell Biol*. 2001; 80:466–478. [PubMed: 11499789]
- Ueda H, Horibe Y, Kim KJ, Lee VH. Functional characterization of organic cation drug transport in the pigmented rabbit conjunctiva. *Invest Ophthalmol Vis Sci*. 2000; 41:870–876. [PubMed: 10711706]
- Uhlen S, Muceniec R, Rangel N, Tiger G, Wikberg JE. Comparison of the binding activities of some drugs on alpha 2A, alpha 2B and alpha 2C-adrenoceptors and non-adrenergic imidazoline sites in the guinea pig. *Pharmacol Toxicol*. 1995; 76:353–364. [PubMed: 7479575]
- Uzunosmanoglu E, Mocan MC, Kocabeyoglu S, Karakaya J, Irkec M. Meibomian Gland Dysfunction in Patients Receiving Long-Term Glaucoma Medications. *Cornea*. 2016; 35:1112–1116. [PubMed: 27055218]
- Whitson JT, Henry C, Hughes B, Lee DA, Terry S, Fechtner RD. Comparison of the safety and efficacy of dorzolamide 2% and brimonidine 0.2% in patients with glaucoma or ocular hypertension. *J Glaucoma*. 2004; 13:168–173. [PubMed: 15097265]
- Whitson JT, Ochsner KI, Moster MR, Sullivan EK, Andrew RM, Silver LH, Wells DT, James JE, Bosworth CF, Dickerson JE, et al. The safety and intraocular pressure-lowering efficacy of brimonidine tartrate 0.15% preserved with polyquaternium-1. *Ophthalmology*. 2006; 113:1333–1339. [PubMed: 16877072]
- Xiong Y, Collins QF, An J, Lupo E Jr, Liu HY, Liu D, Robidoux J, Liu Z, Cao W. p38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. *J Biol Chem*. 2007; 282:4975–4982. [PubMed: 17172644]
- Zhang Y, Kam WR, Liu Y, Chen X, Sullivan DA. Influence of Pilocarpine and Timolol on Human Meibomian Gland Epithelial Cells. *Cornea*. 2017; 36:719–724. [PubMed: 28476050]

Highlights

- Brimonidine stimulates the differentiation of human meibomian gland epithelial cells
- Brimonidine decreases the proliferation of, and Akt and p38 signaling in, these cells
- Brimonidine's cellular effects are duplicated by clonidine, but not by phenylephrine
- Brimonidine enhances the cellular content of sterol regulatory binding protein-1

Figure 1.

Impact of brimonidine on proliferation and p-AKT signaling in iHMGECs. (A, B) Cells were cultured in KSFM with brimonidine for 7 days and counted with a hemocytometer. Cell rounding and detachment began to occur within 3 days in the 500 µg/ml brimonidine group. (C) Cells were grown in KSFM supplemented with EGF and BPE for 2 days, starved in KSFM overnight, and incubated with brimonidine for 15 minutes prior to lysis and immunoblotting. Band intensity was normalized to β -actin and analyzed using ImageJ. * $P < 0.05$ and ** $P < 0.005$. Data are shown as mean $\pm$ standard error. One representative result of 3 total experiments is shown.

Figure 2.

Effect of brimonidine, clonidine and phenylephrine on lysosome and neutral lipid accumulation in iHMGECs. Cells were treated in DMEM/F12 containing 10% FBS with or without drugs for 5-7 days. Cells were then stained for lysosomes (LysoTracker Red DND-99) and neutral lipids (HCS LipidTOX Green) and investigated under a fluorescent microscope. Azithromycin was used as a positive control. The results shown are from a single representative of 3 experiments. Scale bar indicates 50 µm.

Figure 3.

Influence of brimonidine, clonidine and phenylephrine on phospholipid accumulation in iHMGEs. Cells were treated with or without α agonists in the presence of a phospholipidosis detection reagent for 6 days. Blue indicates lysosomes, green presents neutral lipids, and red demonstrates phospholipids. Azithromycin was used as a positive control. The results shown are from single representative of at least 2 experiments with each treatment. Scale bar indicates 50 μ m.

Figure 4.

Impact of brimonidine, clonidine and phenylephrine on signaling pathways. (A) Cells were maintained in DMEM/F12 containing 10% FBS for 6 days, serum-starved overnight, then treated with drugs for 15 minutes prior to lysis and immunoblot. Band intensity was normalized to the loading control (β -actin/ERK2/P38/JNK) and analyzed using ImageJ. (B) Cells were grown in KSFM overnight, stimulated with drugs for 15 minutes, lysed, and assayed for cAMP accumulation. * $P < 0.05$ and ** $P < 0.005$. One representative result of 3 experiments is shown as mean $\pm$ standard error.

Figure 5.

Effect of brimonidine on the expression of SREBP-1, LAMP-1 and LC3A in iHMGECs. Cells were treated with brimonidine for 6 days in DMEM/F12 containing 10% FBS and evaluated by immunoblot. Band intensity was normalized to β -actin and analyzed using ImageJ. One representative result of at least 2 experiments is shown as mean $\pm$ standard error. * $P < 0.05$ and ** $P < 0.005$.

Figure 6.

Influence of RX821002 and MK912 on lysosome and neutral lipid accumulation in iHMGECs. Cells were treated in DMEM/F12 containing 10% FBS with drugs for 5 (A, B) or 2 (C) days. Cells were stained with LysoTracker Red DND-99 and HCS LipidTOX Green neutral lipid for lysosomes and neutral lipids. Merged images show the co-localization of lysosome and neutral lipid. The results shown are from one of at least 2 experiments. Scale bar indicates 50 µm.

Table 1Affinities of α adrenergic agonists and antagonists for α adrenergic receptor subtypes

Compound	Receptor binding affinity (K _i , nM)		
	α 2A	α 2B	α 2C
α 2 Adrenergic agonist			
Brimonidine	3.7 ± 0.8	512 ± 3	120 ± 13
Clonidine	11 ± 3	40 ± 4	134 ± 4
α 2 Adrenergic antagonist			
RX821002	0.14 ± 0.01	7.9 ± 0.8	1.1 ± 0.1
MK912	0.40 ± 0.03	8.3 ± 0.4	0.08 ± 0.01

The α 2A, α 2B and α 2C are adrenergic receptor subtypes. The K_i is an inhibitor constant, and equal to the concentration necessary to produce half maximum inhibition. The listed concentrations are from Uhlen *et al.* [44] and Lomasney *et al.* [45]. For comparison, phenylephrine has affinities for α 1A (1,400), α 1B (23,900) and α 1C (47,800) receptors [46].