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β 2-Adrenergic Receptor Signaling Mediates Corneal Epithelial Wound Repair

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Abstract

Purpose— β -Adrenergic receptor (AR) antagonists are frequently prescribed ophthalmic drugs, yet previous investigations into how catecholamines affect corneal wound healing have yielded conflicting results. With the use of an integrated pharmacologic and genetic approach, the authors investigated how the β -AR impacts corneal epithelial healing.

Methods—Migratory rates of cultured adult murine corneal epithelial (AMCE) cells and in vivo corneal wound healing were examined in β 2-AR^{+/+} and β 2-AR^{-/-} mice. Signaling pathways were evaluated by immunoblotting.

Results—The β -AR agonist isoproterenol decreased AMCE cell migratory speed to 70% of untreated controls, and this was correlated with a 0.60-fold decrease in levels of activated phospho-ERK (P-ERK). Treatment with the β -AR antagonist (timolol) increased speed 33% and increased P-ERK 2.4-fold ($P < 0.05$). The same treatment protocols had no effect on AMCE cells derived from β 2-AR^{-/-} mice; all treatment groups showed statistically equivalent migratory speeds and ERK phosphorylation. In β 2-AR^{+/+} animals, the β -AR agonist (isoproterenol) delayed the rate of in vivo corneal wound healing by 79%, whereas β -AR antagonist (timolol) treatment increased the rate of healing by 16% ($P < 0.05$) compared with saline-treated controls. In contrast, in the β 2-AR^{-/-} mice, all treatment groups demonstrated equivalent rates of wound healing. Additionally, murine corneal epithelial cell expressed the catecholamine-synthesizing enzyme tyrosine hydroxylase and detectable levels of epinephrine (184.5 pg/mg protein).

Conclusions—The authors provide evidence of an endogenous autocrine catecholamine signaling pathway dependent on an intact β 2-AR for the modulation of corneal epithelial wound repair.

β -Adrenergic receptor (AR) antagonists are among the most frequently prescribed ophthalmic drugs, and these topical β -blockers are among the most common treatments for ocular hypertension and glaucoma.^{1–3} Although the corneal epithelium expresses some α 1 adrenoceptors, the predominantly expressed receptor in bovine, rabbit, and human eyes is β 2-AR.^{4–7} Catecholamine ligands for these receptors are ubiquitously found in the eye as well.

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For example, norepinephrine is found in the aqueous humor (0.796 ng/mL)⁸ and in tears (4.4 nM),⁹ as is epinephrine (3.9 nM in tears).⁹ These may derive in part from the sympathetic nerves that originate in the superior cervical ganglion and traverse the corneal stroma to sparsely penetrate the corneal epithelium, as has been detected in human and other mammalian corneas.^{10–13} An additional source of catecholamines to the cornea may be through diffusion from the limbal and conjunctival sympathetic plexus, which is associated with blood vessels.^{10,12} Lastly, using competition assays with [³H] dihydroalprenolol, the presence of β -AR in rabbit corneal epithelial cell culture has been confirmed, and isoproterenol, which results in the stimulation of cyclic adenosine monophosphate (cAMP), has been identified as a more potent adrenergic agonist than epinephrine or norepinephrine.¹⁴

Investigations into how catecholamines affect corneal epithelial proliferation and wound healing have yielded conflicting results. For instance, β -AR antagonists have been reported to delay^{15,16} or enhance¹⁷ corneal epithelial wound healing, and β -agonists have been shown to stimulate the proliferation and migration of transformed corneal epithelial cells.¹⁷ In addition, using electrodes implanted in the left cervical sympathetic nerve in rabbits, it has been shown that chronic sympathetic stimulation *in vivo* significantly decreases the epithelial migration rate and increases wound closure time.¹⁸

Recent work in our laboratory has shown that β -AR agonists decrease epithelial keratinocyte cell migratory speed and retard *in vitro* and *ex vivo* skin wound healing by decreasing activation of the promigratory extracellular signal-regulated kinase (ERK).^{19–23} Furthermore, in cultured keratinocytes, we have shown that isoproterenol β -AR-mediated inhibition of migratory speed is not through a cAMP-dependent mechanism but rather is mediated through inhibition of the ERK mitogen-activated protein (MAP) kinase signaling.¹⁹ β -AR antagonists have the opposite effect, increasing cell migratory speed and increasing the activation of ERK.^{19–23} Published studies have demonstrated that β -AR agonists also decrease cell migratory speed and ERK activation in adult human corneal epithelial cells.^{20,23} These studies of the β -AR agent's effect on wound healing illustrates that keratinized vascular epithelium of skin and nonkeratinized avascular epithelium of cornea may exhibit similar mechanisms of wound healing.

The question that remains unanswered in pharmacologic studies using β -AR agents is whether the effects observed on corneal epithelial wound healing are secondary to receptor-mediated signaling, either through the β 2-AR or another sub-type of β -AR, or whether they are secondary to an extra-receptor-mediated mechanism. We present evidence using biochemical, cell biology, and genetic approaches that indicate the β 2-adrenergic signaling pathway is a potent modulator of corneal epithelial cell migration *in vitro* and of corneal epithelial wound healing *in vivo*. We also provide data that epithelial-derived catecholamines are produced by and may play an autocrine role in epithelial function.

Materials and Methods

Murine Corneal Epithelial Cell Growth

Animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research, under protocols approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Using a modification of the protocol described by Tseng and colleagues,²⁴ adult murine corneal epithelial (AMCE) cells were isolated and cultured from male β 2-AR^{-/-} FVB/NJ mice (kind gift of Brian Kobilka, Stanford University²⁵) and male β 2-AR^{+/+} FVB/NJ congenic controls (stock no. 001800; Jackson Laboratory, Bar Harbor, ME). The genotypes of the β 2-AR^{+/+} and β 2-AR^{-/-} mice were confirmed by PCR using published primers.²⁵

After subjecting mice to CO₂ asphyxiation, 10 murine globes were enucleated and were washed three times in Dulbecco phosphate-buffered saline (D-PBS) with 3% antibiotic-antimycotic solution (10,000 U penicillin, 10,000 μ g streptomycin, 25 μ g amphotericin B/mL; Gibco [Grand Island, NY]). Cells were cultured in murine corneal growth medium (mCGM) (EpiLife HCGS medium [Cascade Biologics, Portland, OR]) with 0.02 mM Ca²⁺, cholera toxin subunit A 10⁻¹⁰ M (Sigma-Aldrich, St. Louis, MO), 10 ng/mL mEGF (Sigma), and 1% antibiotic-antimycotic (Gibco). Mouse eyes were then incubated at 4°C for 18 hours in mCGM containing 15 mg/mL dispase II (Roche Diagnostics, Indianapolis, IN) and 100 mM sorbitol (Sigma).²⁴ The easily separated corneal-limbal epithelial sheets were dissociated in 0.25% trypsin with EDTA (Gibco) at 37°C for 10 minutes, and the resultant single-cell suspension was plated at a density of 2×10^4 cells/cm² on plastic cell culture dishes (Falcon Labware; BD Biosciences, San Jose, CA) precoated for 1 hour at 37°C with 60 μ g/mL bovine dermal collagen (Cohesion Technologies, Palo Alto, CA) in D-PBS.

Cells were grown in mCGM at 37°C in a humidified atmosphere of 5% CO₂. Once they were 80% confluent, AMCE cells were subcultured first by treatment with 0.05% EDTA (USB Corp., Cleveland, OH) for 5 minutes and then by the addition of 0.1% trypsin for 5 minutes, both at 37°C. The trypsin solution was subsequently neutralized using a bovine serum-containing medium.

Cell Migration Assay

Analysis of the locomotory speed of individual cells was performed, as previously described.^{23,26} Glass-bottom culture dishes (MatTek Corp., Ashland, MA) were collagen coated using a 60 μ g/mL solution of bovine dermal collagen in D-PBS for 1 hour at 37°C. AMCE cells (passage 1; 2×10^4 cells in 200 μ L mCGM) were plated in the 14-mm glass microwells for 1 hour at 37°C in a humidified atmosphere of 5% CO₂. An additional 1 mL mCGM was then added to the dish, and the cells were further incubated for 1 hour at 37°C. The medium was then exchanged with 1 mL mCGM containing 1 μ M isoproterenol HCl (β -AR agonist; Calbiochem, San Diego, CA), 20 μ M timolol (β -AR antagonist; Sigma), or no added drug. Culture dishes were placed in a 37°C incubator on an inverted microscope (Diaphot; Nikon, Tokyo, Japan), and time-lapse images of the cell positions were digitally captured every 10 minutes over a 1-hour period using cameras (Retiga-EX; Q-Imaging, Burnaby, BC, Canada) controlled by a custom automation written in software (Open Laboratory; Improvision, Lexington, MA) on a computer (Macintosh G4; Apple, Cupertino, CA). After the center of mass of each cell was tracked using the software (Open Laboratory; Improvision), migration speed and distance were calculated and imported to a spreadsheet (Excel; Microsoft, Redmond, WA). Statistical significance between the means of two cell populations was calculated using the Student's *t*-test with *P* < 0.05.

In Vivo Murine Corneal Wound Healing

In vivo corneal wound-healing experiments were completed as previously described.²⁷ Male β 2-AR^{+/+} and β 2-AR^{-/-} mice underwent general anesthesia using an intraperitoneal injection of a 50 mg/kg ketamine + 5 mg/kg xylazine solution and were provided general analgesia using subcutaneous injection of 0.05 mg/kg buprenorphine (Western Medical Supply, Los Angeles, CA). A 2-mm diameter, circular, axial corneal epithelial defect was created using a 2-mm ophthalmic crescent blade (Grieshaber, Schaffhausen, Switzerland) and a micro hoe (Sloane LASEK; Katena, Denville, NJ). The corneal wound was stained with a 1 mg/mL solution of fluorescein (Akorn, Buffalo Grove, IL) in balanced salt solution (BSS; Alcon, Fort Worth, TX) and was immediately magnified by a stereomicroscope (Nikon SMZ-10A; Technical Instruments, San Francisco, CA) illuminated with a 16-LED, 464-nm flashlight (LDP, Carlstadt, NJ) and photographed. The corneal wound was then treated twice daily with 1 drop of a neomycin, polymyxin B, and gramicidin antibiotic solution (Bausch & Lomb, Tampa,

FL), followed by 25 μ L BSS alone, 1% isoproterenol in BSS (every 4 hours), or 0.5% timolol in BSS (twice per day). β 2-AR^{+/+} and β 2-AR^{-/-} mice were tested side by side for each treatment group. Corneal wound healing was monitored approximately 8 hours after deepithelialization and every 4 hours thereafter until the wound healed.

The rate of healing of the corneal wound was analyzed, as previously described, by adapting formulas used by Crosson, estimating the murine corneal radius of curvature (R) as 1.414 mm.²⁸ The analyst was masked to the genotype and the treatment used.^{27,29} The rate of wound healing was expressed as a decrease per unit time in the wound radius (r) of the circle equivalent to surface area A s. As previously described, graphing wound radius r (mm) versus time (h) reveals an initial latent phase, followed by a linear phase of wound healing.²⁹ Using the XY scatter plot of the linear phase of healing for each treatment group, linear regression analysis was performed (Minitab 14 Statistical Software; MiniTab Inc., State College, PA), the slope of which revealed the rate of wound healing (μ m/h). Statistical significance was determined using the F -test with $P < 0.05$.

Immunoblot for Activation of ERK and for Identification of Tyrosine Hydroxylase

Confluent AMCE cells from β 2-AR^{+/+} and β 2-AR^{-/-} male mice on a Costar six-well plate were incubated with mCGM alone (control), mCGM containing 1 μ M isoproterenol, or mCGM containing 20 μ M timolol for 5 minutes in two independent experiments. AMCE cells were placed immediately on ice, washed twice with ice-cold PBS containing inhibitors (50 mM NaF and 1 mM Na₃VO₄), and scraped in 40 μ L lysis buffer (PBS containing 0.5% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10 μ g/mL leupeptin, 30 μ g/mL aprotinin, 200 μ g/mL phenylmethylsulfonyl fluoride, 10 μ g/mL pepstatin A). Lysates were incubated on ice for 20 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4°C. The protein concentration of the samples was determined using the Bradford Assay (Bio-Rad Laboratories, Hercules, CA). Supernatants were stored at -80°C until they were used for electrophoresis. Five micrograms (phospho-ERK [P-ERK] blot) or 35 μ g (tyrosine hydroxylase blot) of each protein sample was added to an equal volume of 2 $\times$ reducing sample loading buffer (0.0625 M Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 5% β -mercaptoethanol) and electrophoresed on 10% polyacrylamide Bis-Tris gels (Bio-Rad Laboratories). Proteins were transferred to Immobilon (Millipore, Billerica, MA) membranes. For the P-ERK blot, the membrane was immunoblotted with an anti-extracellular signal-related kinase (ERK) antibody (9102; Cell Signaling Technology, Beverly, MA) and an anti-phospho-ERK antibody (9101; Cell Signaling Technology). For the tyrosine hydroxylase (TH) blot, the membrane was immunoblotted with an anti-TH antibody (AB152; Chemicon, Temecula, CA). Immunoblots were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry was performed on scanned images using a gel-plotting macro in NIH Image 1.62. Data were normalized so that the average of the mean phosphorylated ERK signal (42 kDa) divided by the mean ERK signal for controls was set at 1, where statistical significance was determined using the Student's t -test with $P < 0.01$.

Enzyme Immunoassay for Quantitative Determination of Epinephrine Levels

After CO₂ asphyxiation, corneal epithelial sheets from the eyes of 60 β 2-AR^{+/+} and 30 β 2-AR^{-/-} adult male mice were isolated by mechanical debridement using an ophthalmic crescent blade or by enzymatic isolation at 4°C for 18 hours using mCGM containing 15 mg/mL dispase II and 100 mM sorbitol, as described. Corneal epithelia were immediately transferred to RIPA buffer (Sigma) at 4°C, containing 0.01% Triton X-100, 0.01 N HCl, 10 μ g/mL leupeptin, 30 μ g/mL aprotinin, 200 μ g/mL phenylmethylsulfonyl fluoride, and 10 μ g/mL pepstatin A, sonicated three times for 15 seconds (Sonicator 250; Branson, Danbury, CT), and stored at -80°C. Lysates were tested in triplicate in an epinephrine enzyme immunoassay (EIA; Biosource, Camarillo, CA), as previously described.²³ A set of standards (0, 1.1, 3.9, 16, 61,

and 283 ng/mL epinephrine) was included, and corneal growth medium supplement (HCGS; Cascade Biologics) was additionally used as a control. The reaction was monitored at 450 nm on a spectrophotometer (Spectramax 340PC; Molecular Devices Corp., Sunnyvale, CA). Sample concentrations were calculated from the linear curve fitted with the standard concentration values. The protein concentration in each extract was calculated using the Bradford assay (Bio-Rad Laboratories), and epinephrine concentrations were reported per milligram of protein in the extract.

Results

Superficial Corneal Wounding Is Confined to the Epithelium

The use of an ophthalmic crescent blade and a LASEK micro hoe for in vivo corneal wounding resulted in the removal of the corneal epithelium with minimal disruption of the basement membrane or underlying stroma, as demonstrated by light microscopy of a toluidine blue-stained mouse cornea (Fig. 1). Therefore, this method of deepithelialization can produce reproducible wounds, such that the rate of change in wound radius per unit time can be compared among treatment groups, which is consistent with previous descriptions of corneal epithelial abrasion methods.^{27,30}

β 2-AR Is Required for β -AR–Mediated Modulation of Corneal Cell Migration

Treatment of AMCE cells derived from β 2-AR^{+/+} mice with a β -AR agonist (isoproterenol) resulted in a 70% decrease in average speed (μ m/min) compared with control untreated cells ($P < 0.001$), whereas those treated with a β -AR antagonist (timolol) exhibited a 33% increase in the average speed ($P < 0.001$; Fig. 2). However, when the same treatment protocols were applied to AMCE cells derived from β 2-AR^{-/-} mice, all treatment groups showed statistically equivalent migratory speeds ($P > 0.05$; Fig. 2).

β 2-AR Is Required for the Delay of In Vivo Corneal Epithelial Wound Healing by a β -AR Agonist and the Acceleration of Healing by a β -AR Antagonist

Representative images of fluorescein-stained β 2-AR^{+/+} and β 2-AR^{-/-} corneas treated with BSS, isoproterenol, or timolol are shown in Figure 3A. When examining wound healing measured by wound radius (mm) versus time (hours), a biphasic process of healing is revealed, as previously reported by Crosson et al.,²⁹ first as a latent phase (0–8 hours) and second as a linear healing phase (more than 8 hours). The latent phase was similar in all groups of animals tested. In contrast, however, there were marked differences in the linear healing phase between the treated and untreated groups. In β 2-AR^{+/+} mice, treatment with a β -AR agonist (isoproterenol) delayed the rate of wound healing by 79% ($P < 0.001$), whereas β -AR antagonist (timolol) treatment increased the rate of healing by 16% ($P < 0.05$; Figs. 3B, 3D). On the other hand, in β 2-AR^{-/-} mice, all treatment groups demonstrated equivalent rates of wound healing ($P > 0.05$; Figs. 3C, 3D). In comparing the untreated mice (control), the β 2-AR^{-/-} mice healed 12% more quickly than mice that expressed the β 2-AR receptor ($P < 0.05$; Fig. 3D). In examining the fitness of the linear regression analysis of each treatment group, we found $R^2 > 0.90$ for all groups except for isoproterenol-treated β 2-AR^{+/+} mice (0.46) and timolol-treated β 2-AR^{-/-} mice (0.87).

Deletion of β 2-AR Interrupts the Modulation of ERK by β -AR

ERK plays a pivotal role in promigratory signaling pathways,^{31,32} is critical for the healing of scratch wounds in confluent monolayers of lens epithelium³³ and corneal epithelial cells,^{34,35} and is phosphorylated within 1 hour of rat corneal wounding.³⁶ Because the β -AR agents altered the migratory rate in corneal epithelial cells, we examined the effect these agents would have on the promigratory ERK. In cultured β 2-AR^{+/+} murine corneal epithelial cells, a β -AR

agonist (isoproterenol) decreased the activation and phosphorylation of ERK (i.e., 42 kDa P-ERK) by 0.60-fold compared with control within 5 minutes of treatment, whereas a β -AR antagonist (timolol) increased ERK phosphorylation (i.e., 42 kDa P-ERK) by 2.4-fold. However, when $\beta 2$ -AR^{-/-} AMCE cells are similarly treated with agonist or antagonist, ERK phosphorylation was not affected (Figs. 4A, 4B).

Murine Corneal Epithelial Cells Synthesize Epinephrine

Even in the absence of exogenously added β -adrenergic agonist, in vivo corneal wound healing was slower in animals that expressed the β -AR receptor than in those without the receptor (Fig. 3D). The addition of a β -AR antagonist to cultured corneal epithelial cells from $\beta 2$ -AR^{+/+} mice, in which neural and lacrimal sources of catecholamines are absent, significantly increased their migratory rate (Fig. 2). These findings suggest that an endogenous catecholamine agonist may be generated by corneal epithelial cells themselves in the in vitro-cultured cells and in the in vivo-wounded cornea and that this endogenous catecholamine may slow migratory rates and healing by virtue of its local autocrine activation of the $\beta 2$ -AR. To test for this possibility, we examined the murine corneal epithelium for the presence of the rate-limiting enzyme necessary for catecholamine production, tyrosine hydroxylase. Immunoblot analysis of lysates from cultured murine corneal epithelial cells of $\beta 2$ -AR^{+/+} and $\beta 2$ -AR^{-/-} mice confirmed the presence of tyrosine hydroxylase (Fig. 5A). Further, we examined whether epinephrine, the preferred agonist of the $\beta 2$ -AR, was present in corneal epithelial cells. Corneal epithelial lysates from $\beta 2$ -AR^{+/+} mice isolated by mechanical deepithelialization demonstrated 184.5 pg epinephrine/1 mg protein (SEM, 79.8), which is significantly higher than the 2.6 mg epinephrine/1 mg protein (SEM, 0.9) found in the corneal growth medium supplement HCGS ($P < 0.05$; Fig. 5B). Additionally, corneal epithelial lysates obtained by dispase digestion and lysates from $\beta 2$ -AR^{-/-} mice showed statistically equivalent levels of epinephrine than did the lysates of $\beta 2$ -AR^{+/+} mice obtained by mechanical deepithelialization (results not shown).

Discussion

According to a study from the United Kingdom, 32% of patients with open-angle glaucoma are treated with timolol.³⁷ Given that timolol, a nonselective $\beta 1$ - and $\beta 2$ -AR antagonist, is one of the most commonly prescribed topical ophthalmic medications, many investigations into its effects on the cornea have been undertaken and have yielded conflicting results. We applied an integrative approach, using both pharmacologic and genetic tools, to determine the effect of β -adrenergic agonists and antagonists on corneal epithelial cell migration and wound healing. We found that an agonist decreased both in vitro corneal epithelial cell migratory speed and in vivo corneal wound healing, whereas a β -AR antagonist increased these parameters, with a correlation to levels of ERK phosphorylation. Additionally, we found that β -adrenoceptor agents cannot exert their effects on cell migration and corneal wound healing after the deletion of the $\beta 2$ -adrenoceptor. Finally, we demonstrate evidence of an endogenous catecholamine- β adrenergic signaling pathway that may modulate corneal epithelial wound repair by autocrine stimulation of the receptor.

The activation of PP2A by β -AR agonists has been demonstrated in cultured adult human corneal epithelial (AHCE) cells, resulting in a dephosphorylation of the promigratory kinase ERK and a decreased migratory speed.²⁰ Additionally, when AHCE cells are treated with okadaic acid at concentrations specific for the inhibition of PP2A, the retardation of migratory speed by a β -AR agonist is reversed, suggesting that $\beta 2$ -AR activation modulates migration by way of PP2A.²⁰

We demonstrate that a similar mechanism of $\beta 2$ -AR modulation of cell migration may exist in murine corneal epithelial cells. $\beta 2$ -AR^{+/+} AMCE cells treated with a β -AR agonist showed a decrease, whereas β -AR antagonist treatment resulted in an increase, in activated P-ERK (Fig.

4A). Using a genetic approach, we demonstrate that when the same treatments were performed on $\beta 2\text{-AR}^{-/-}$ AMCE cells, no modulation of ERK was observed. Furthermore, levels of P-ERK in the corneal epithelium of $\beta 2\text{-AR}^{-/-}$ mice are increased compared with those of $\beta 2\text{-AR}^{+/+}$ mice, irrespective of β -adrenoceptor agent treatment. This finding may be attributed to constitutive increases in P-ERK because of decreased $\beta 2\text{-AR}$ signaling and decreased PP2A activity in $\beta 2\text{-AR}^{-/-}$ mice. The present work extends previous findings to demonstrate the requirement of a functional $\beta 2$ -adrenoceptor for the modulation of ERK phosphorylation in response to pharmacologic β -adrenergic agents.

The rate of corneal wound healing in the $\beta 2\text{-AR}^{-/-}$ mice receiving only saline (BSS) was significantly higher than that of the similarly treated $\beta 2\text{-AR}^{+/+}$ mice (Fig. 3D). Thus, even in the absence of exogenously added β -AR agonist, corneal epithelia that express the $\beta 2\text{-AR}$ receptor exhibit slower wound repair than those in which the receptor has been genetically deleted. If one presumes that no significant unknown genetic differences related to migration exist between $\beta 2\text{-AR}^{+/+}$ and $\beta 2\text{-AR}^{-/-}$ FVB/NJ mice, then these results suggest an endogenous activator may be present in the wounded cornea. Alternatively, adrenal gland-generated catecholamines could be delivered by the circulation to the wounded eye³⁸ or they could be delivered by tears from lacrimal gland-generated catecholamines. Indeed, norepinephrine is found in tears (4.4 nM), as is epinephrine (3.9 nM).⁹ Sparse adrenergic nerves are found in the cornea as well and could also contribute to the local generation of catecholamines.^{11–13} However, adrenal and lacrimal sources of catecholamines do not explain how the addition of a β -AR antagonist to cultured corneal epithelial cells from $\beta 2\text{-AR}^{+/+}$ mice significantly increased their migratory rate because these elements are not present in the culture. Furthermore, the corneal growth medium supplement used for cell cultivation contains only 2.6 pg epinephrine/1 mg protein.

Another possible source of catecholamines in the cornea could be the corneal epithelial cells themselves. Human keratinocytes express the enzymes tyrosine hydroxylase and phenylethanolamine-*N*-methyl transferase required for catecholamine synthesis and, indeed, contain epinephrine in the range of 300 to 900 pg epinephrine/1 mg protein.^{22,39} Here we demonstrate that not only do murine corneal epithelial cells contain the catecholamine-synthesizing enzyme tyrosine hydroxylase, they also contain epinephrine, at an average of 184.5 pg epinephrine/1 mg protein extract, suggesting endogenous synthesis by the corneal epithelial cells. For the immunoblot for tyrosine hydroxylase, lysates from AMCE cell culture were used to avoid neural tissue sources from the cornea. For the EIA of epinephrine, though mechanical deepithelialization might have inadvertently included neural elements or lacrimal gland-derived catecholamines, these possible cofounders were avoided when lysates of dispase II-digested epithelial sheets were analyzed. Further investigation will be required to determine whether the epinephrine found within the corneal cells is indeed endogenously synthesized or whether it represents epithelial uptake of adrenal-, lacrimal-, or neuronal-derived catecholamines. Nevertheless, the finding of 184.5 pg epinephrine/1 mg protein within the corneal epithelium suggests that local levels of β -AR agonist are sufficient to constitutively activate the receptor; thus, the increase in wound healing observed by the addition of a β -blocker could indeed be derived from the blockade of this basal, local migratory retardation by catecholamines.

Past research on the effect of β -AR agonists and antagonists might have yielded conflicting results because of differences in the type of control used, the presence of preservatives, the wounding mechanism, and the cell types examined. For the *in vivo* corneal wound-healing experiments presented, separate mice formed the treatment and control groups so that systemic effects of topical agents would not confound the control group. In addition, the β -adrenergic agents were prepared freshly before each treatment, without the addition of preservatives. Mechanical deepithelialization of the cornea with minimal disruption of the stroma avoided

confounding factors of chemical wounding. Additionally, for the in vitro corneal epithelial experiments, passage 1 cells were used for each experiment, which minimized possible confounding factors of differentiation and transformation.

In conclusion, using an integrative approach that combines pharmacologic studies with genetically modified mice that do not express the β 2-AR, we demonstrated that it is the β 2-AR signaling that mediates the effects of topically applied β -adrenoceptor agonists and antagonists. A β -AR agonist decreased corneal cell migratory speed and in vivo wound healing by activating the β 2-AR, possibly through a mechanism mediated by the ERK signaling cascade. On the other hand, by competitively inhibiting endogenous catecholamines present in the cornea, a β -AR antagonist increased corneal cell migratory speed and wound healing. Because timolol is one of the most commonly used ophthalmic agents used for the treatment of glaucoma, it is critical to understand its effects on the corneal epithelium. Many forms of timolol (e.g., timolol maleate [Timoptic]) contain preservatives such as benzalkonium chloride, which confound many of the clinical observations of how timolol alters corneal physiology. Although it is speculative, the in vivo corneal wound experiments in mice created a model that may parallel the healing of corneal abrasions in humans. In addition, given that timolol increases cell locomotory speed in AHCE cell culture, a possible clinical trial of preservative-free timolol therapy after corneal abrasions, persistent epithelial defects, or recurrent erosions may be warranted. The pharmacologic approach of using timolol maleate in BSS and the genetic approach of using β 2-AR knockout mice will, it is hoped, not only elucidate how timolol affects corneal wound healing but will create a paradigm of how timolol affects epithelial wound healing overall.

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Corneal Wounding Produces an Epithelial Wound

Figure 1.

Superficial corneal wounding is confined to the epithelium. A 2-mm disposable biopsy punch was blotted with methylene blue and was gently depressed against the murine cornea, outlining the zone for deepithelialization. Two-millimeter circular, axial corneal defects were created using a 2-mm crescent blade and a LASEK micro hoe. Dissected corneas ($n = 4$) were embedded in methacrylate resin, cut at a thickness of $1.5 \mu\text{m}$, and stained with toluidine blue.

Figure 2.

$\beta 2\text{-AR}$ is required for β -adrenergic agent-mediated modulation of corneal epithelial cell migration. Passage 1 adult murine corneal epithelial cells from $\beta 2\text{-AR}^{+/+}$ or $\beta 2\text{-AR}^{-/-}$ mice were plated on collagen-coated, glass-bottom culture dishes at a density of 26 cells/ mm^2 in mCGM and were incubated for 2 hours at 37°C. The migration of single cells in the microscopic field of view was measured for a 1-hour period while the cells were in mCGM alone, 1 μM isoproterenol, or 20 μM timolol. Data displayed are the mean $\pm$ SEM of the speed ($\mu\text{m}/\text{min}$) of each individual cell for each treatment group. * $P < 0.001$ compared with control; Student's *t*-test.

Figure 3.

$\beta 2\text{-AR}$ is required for the delay of *in vivo* corneal epithelial wound healing by a $\beta\text{-AR}$ agonist and the acceleration of healing by a $\beta\text{-AR}$ antagonist. Using male $\beta 2\text{-AR}^{+/+}$ ($n = 25$) or $\beta 2\text{-AR}^{-/-}$ mice ($n = 25$), 2-mm diameter circular, axial corneal epithelial wounds were created using a crescent blade and a LASEK micro hoe. Corneas were treated topically with BSS, BSS with 1% isoproterenol every 4 hours, or BSS with 0.5% timolol twice daily, and wound healing was monitored stereomicroscopically using fluorescein staining, as shown (A). The polygonal area (A_p) of the wound was determined using NIH ImageJ, as described (B, C). Values plotted are the rates of wound healing, with standard error of the coefficient illustrated by error bars (D). * $P < 0.05$ using the F -test, compared with congenic, control group $\beta 2\text{-AR}^{+/+}$ mice.

Figure 4.

Deletion of the β 2-AR in corneal epithelial cells interrupts the modulation of ERK signaling by β -adrenergic agents. Lysates of confluent cultured AMCE cells from male β 2-AR $^{+/+}$ and β 2-AR $^{-/-}$ mice that had been treated with mCGM alone (control), 1 μ M isoproterenol (β -AR agonist), or 20 μ M timolol (β -AR antagonist) were electrophoresed and immunoblotted with an anti-ERK antibody and an anti-phospho-ERK (P-ERK) antibody (**A**). Blots from separate experiments were scanned, and densitometry was performed (NIH Image 1.62). Data were normalized so that the average of the mean phosphorylated ERK signal (42 kDa P-ERK) divided by the mean ERK signal for control was set at 1. All data were averaged, statistically analyzed, and represented graphically as a ratio of the control (**B**). Values plotted are mean $\pm$ SEM. * $P < 0.01$ compared with control. Data shown are representative of two independent experiments in duplicate from murine corneal epithelial cells isolated from multiple corneas.

Figure 5.

Murine corneal epithelial cells expressed the catecholamine synthetic enzyme tyrosine hydroxylase and contained epinephrine. Lysates of confluent cultured AMCE cells from $\beta 2$ -AR^{+/+} and $\beta 2$ -AR^{-/-} mice were electrophoresed and immunoblotted with an anti-tyrosine hydroxylase antibody (**A**). Lysates of corneal epithelial sheets from $\beta 2$ -AR^{+/+} and $\beta 2$ -AR^{-/-} mice obtained by mechanical deepithelialization were tested in triplicate in an epinephrine (EPI) enzyme immunoassay. The amount of epinephrine (pg) per milligram protein in the extract was graphically represented $\pm$ SEM (**B**). * $P < 0.05$ compared with HCGS levels of epinephrine.