Carbonic Anhydrase Inhibitors in Corneal Endothelial Transport

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Submitted: October 30, 2013
Accepted: March 13, 2014

Citation: Malikowski TM, Bosch JB, Min S, Duffey ME, Patel SP. Carbonic anhydrase inhibitors in corneal endothelial transport. *Invest Ophthalmol Vis Sci*. 2014;55:2652–2658. DOI:10.1167/iovs.13-13534

Purpose. Carbonic anhydrases play a central buffering role in current models of fluid transport in corneal endothelium, but in humans, clinical use of carbonic anhydrase inhibitors (CAIs) for the management of glaucoma does not cause corneal swelling. This study compares species differences in response to CAIs in human versus bovine corneal endothelial transport.

Methods. Short-circuit current (I_{sc}) measurements were performed on bovine and human corneal endothelium under identical conditions. The effects of four CAIs (acetazolamide, brinzolamide, dorzolamide, and ethoxzolamide) were measured. Endothelial expression of carbonic anhydrase II and IV was evaluated by immunofluorescence microscopy. Functional presence of carbonic anhydrase activity was determined using the Hansson's cobalt sulfide histochemical method.

Results. All four CAIs decreased bovine I_{sc} (% change in I_{sc}: acetazolamide, -21.0 ± 9.5, $n = 8$; brinzolamide, -35.5 ± 13.5, $n = 9$; dorzolamide, -33.6 ± 7.2, $n = 8$; ethoxzolamide, -35.3 ± 12.9, $n = 8$). That decrease was not present in humans (% change in I_{sc}: acetazolamide, 16.2 ± 20.1, $n = 3$; brinzolamide, 6.7 ± 13.9, $n = 3$; dorzolamide, 8.0 ± 20.4, $n = 3$; ethoxzolamide, -4.8 ± 10.3, $n = 2$). Despite no functional effect of CAIs on I_{sc}, both carbonic anhydrase II and IV were present in human corneal endothelium by immunofluorescence microscopy. Histochemical analysis of human corneal endothelium revealed functionally active carbonic anhydrase activity inhibited by brinzolamide.

Conclusions. Carbonic anhydrase facilitates ion transport impacting the corneal endothelial I_{sc} in bovine but not human corneal endothelium, despite its presence and functional activity in human tissue. This finding supports the clinical observation of no corneal swelling in humans administered CAIs and suggests that alternative ion transport mechanisms may be operational in corneal endothelium of different species.

Keywords: corneal endothelium, carbonic anhydrases, ion transport, human

The carbonic anhydrases are a pervasive family of enzymes in eukaryotes and prokaryotes that facilitate the bidirectional conversion of CO_2 and H_2O to H^+ and HCO_3^-. In the corneal endothelium, carbonic anhydrase facilitates fluid transport to maintain corneal stromal deturgescence. The two main models developed from experimentation on rabbit and bovine corneas involve either anion or lactate secretion with obligatory water secretion.[1] In the anion secretion model, HCO_3^- and Cl^- are loaded into the cell across the basolateral membrane through the concerted actions of the Na^+/K^+-ATPase, Na^+/ HCO_3^- cotransporter, Na^+/H^+ exchanger and the Cl^-/ HCO_3^- anion exchanger. Intracellular carbonic anhydrase facilitates HCO_3^- loading. At the apical membrane, HCO_3^- and/or Cl^- leave the cell through anion-selective channels. In the lactate transport model, fluid movement is coupled to the bulk transport of lactate from corneal stroma to aqueous humor via endothelial monocarboxylate transporters. The monocarboxylate transporters couple lactate transport with H^+. Intracellular carbonic anhydrase facilitates buffering of the H^+. Both of these models support the finding of corneal swelling on application of carbonic anhydrase inhibitors (CAIs) in rabbit corneas both in vitro and in vivo.[2–4]

These models of carbonic anhydrase involvement in corneal endothelial fluid transport developed from animal studies fail to account for a common human clinical observation. Carbonic anhydrase inhibitors are used frequently for the treatment of glaucoma, but corneal endothelial function is rarely disrupted.[5–7] Corneal swelling in response to CAIs has been noted under conditions of endothelial compromise, such as with corneal guttata.[7] Although there are several case reports of CAIs causing corneal edema, those corneas were also under additional stress of endothelial compromise from conditions

such as low endothelial cell density, prior penetrating keratoplasty with allograft rejection, or uveitis.[8–11] Although carbonic anhydrases have been identified in human corneal endothelium, their functional roles in normal corneal physiology are unknown.[12]

To decipher the physiologic role of carbonic anhydrase, in this study, we evaluate the effects of four CAIs on human and bovine corneal endothelium. We use the Ussing chamber short-circuit current technique with fresh bovine and human corneal tissue. We find significant species differences in the effects of the CAIs. Our findings support the basic clinical observation in humans that CAIs do not alter net fluid transport in the cornea.

Methods

Human and Bovine Tissue

Research protocols were approved by the Research and Development Committee of the Veterans Affairs Western New York Healthcare System Medical Center (Buffalo, NY, USA). Human corneas not suitable for transplantation stored in Optisol GS (Bausch and Lomb, Rochester, NY, USA) or Eusol (Alchimia, Padova, Italy) at 4°C were procured from Upstate New York Transplant Services (Buffalo, NY, USA). Bovine eyes were procured from regional abattoirs, brought to the laboratory on ice and used for experimentation within 12 hours postmortem.

Solutions and Drugs

The normal physiologic solution used for recordings was a modified Ringer's solution consisting of (in mM) the following: 111.6 NaCl, 29 $NaHCO_3$, 4.8 KCl, 1.0 $CaCl_2$, 0.8 $MgCl_2$, 0.9 NaH_2PO_4, 10 HEPES, and 5 glucose, bubbled with 5% CO_2/95% air, at pH 7.5. Bicarbonate-free Ringer's solution was prepared by omitting $NaHCO_3$, increasing HEPES to 20 mM and bubbling with 100% air with pH adjusted to 7.5. Addition of HCO_3^- (as $NaHCO_3$) up to 12 mM to this solution did not appreciably change pH. Drugs were as follows: acetazolamide, brinzolamide, ethoxzolamide, and ouabain, purchased from Sigma-Aldrich (St. Louis, MO, USA), and dorzolamide was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Using Chamber Short-Circuit Current Instrumentation

Short-circuit current experiments were performed with the EasyMount Ussing chamber system (P2300 chambers; Physiologic Instruments, Inc., San Diego, CA, USA). Measurements were acquired (VCC MC6 Multichannel Voltage/Current Clamp; Physiologic Instruments, Inc.) and digitized under the control of Acquire and Analyze software (Physiologic Instruments, Inc.) run on a personal computer. Modifications were made to optimize short-circuit current (I_{sc}) measurements from the low-resistance corneal endothelial tissue.[13] The current-passing and voltage-sensing electrodes placed within the recording chamber were gel-loading pipette tips cut with a long bevel to fit within the chamber. The tips were plugged with 3% agar prepared in Ringer's solution and back-filled with Ringer's solution. The current-passing electrodes were Ag/AgCl. The voltage-sensing tips connected to tubing filled with Ringer's solution mating with 3-cm bridges of 3% agar in Ringer's solution and then tubing filled with 3-M KCl connecting to calomel electrodes. To establish stable salt gradients, the apparatus was equilibrated at least 12 hours before experimentation.

Tissue Preparation

Bovine corneal tissue was prepared as follows. A 360° conjunctival peritomy was performed to expose bare sclera around the limbus. The anterior chamber was injected by the limbus with Ringer's solution to achieve a modestly firm IOP to allow subsequent removal of the epithelium by mechanical scraping with a surgical blade. The corneoscleral button was then removed following a 360° scleral incision approximately 4 mm posterior to the limbus. The corneoscleral button was placed endothelial-side up in a Petri dish and kept moist with Ringer's solution. A central square of corneal endothelium with attached stroma was cut with razor blades and placed into the Ussing chamber tissue holder with a small ring of silicone grease on the stromal side and a silicone o-ring on the endothelial side to minimize edge damage. The recording aperture of the holder was 0.30 cm^2.

Human corneal tissue was removed from the storage solution and equilibrated through two Petri dishes of prewarmed Ringer's solution over approximately 15 minutes. In the interim, the cornea was briefly mounted on an artificial anterior chamber (Katena Products, Inc., Denville, NJ, USA) filled with Ringer's solution to facilitate mechanical removal of the epithelium by scraping with a surgical blade. Initial experiments were performed by mounting the human cornea on the 0.30 cm^2 holder used for bovine experimentation by removal of the scleral rim and placement of peripheral relaxing incisions to allow the tissue to flatten in the holder. Subsequent experiments used a holder specifically machined to accommodate the curvature of the cornea and scleral rim (obviating the need for relaxing incisions), allowing a 0.85 cm^2 recording aperture.

Short-Circuit Current Recordings and Data Analysis

The prepared tissues were placed within the Ussing chambers and mounted on a heating block stand. Each chamber was filled with 5 mL Ringer's solution maintained at 35 to 36°C. Bubble lift with humidified 5% CO_2/95% air (or 100% air for bicarbonate-free Ringer's) allowed mixing and gassing of the solutions in the chambers. Tissues were voltage-clamped and equilibrated for 20 to 30 minutes until stable plateaus in I_{sc} values were achieved. Selected drugs were then added to the chamber facing the apical side of the endothelium. Drug additions were separated by at least 10 minutes. Percent drug effect was determined by measuring the change in I_{sc} from initial plateau to 10 minutes after drug addition, dividing by the total I_{sc} (initial plateau minus final value after ouabain addition) and multiplying by 100. Although I_{sc} approached zero following ouabain addition, we noted systematic variability between the recording chambers with greater offset in one compared with the other under identical conditions. Because the corneal endothelium is a low-resistance tissue with small I_{sc}, small drifts in electrode junction potentials lead to small differences in electrode asymmetry during experiments. Therefore, we calculated a mean and SD of final I_{sc} offset value following ouabain addition for more than 60 consecutive experiments for each set of electrodes. Only data falling within 1 SD of the mean for each electrode pair was included in quantitative data analysis.

Immunofluorescence Localization

Human corneas were incubated in Ringer's solution at 37°C for 30 minutes followed by fixation in 3% paraformaldehyde and mounting in optimal cutting temperature (OCT) embedding medium (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA,

Table 1. Characteristics of Human Donor Corneal Tissue

Donor Age, y	Death to Preservation, h:min	Death to Use, d	Lens Status	Reason Not Used for Transplantation	Storage Medium	Cause of Death
17	4:31	8	Phakic	Lid-line infiltrates	Optisol-GS	Gunshot wound to head
25	8:41	6	Phakic	Peripheral infiltrate	Optisol-GS	Anoxic encephalopathy
58	10:31	7	Phakic	Positive blood culture	Optisol-GS	Metastatic breast cancer
66	12:00	5	Phakic	Peripheral infiltrate	Optisol-GS	Cerebrovascular accident
68	8:33	6	Phakic	Positive blood culture	Optisol-GS	Pneumonia
69	9:31	6	Phakic	Positive blood culture	Optisol-GS	End-stage renal disease
72	10:23	6	Pseudophakic	Peripheral infiltrates	Optisol-GS	Chronic obstructive pulmonary disease
72	8:45	12	Pseudophakic	Sepsis	Optisol-GS	Cerebrovascular accident
76	3:13	5	Phakic	Positive blood culture	Eusol-C	Cerebrovascular accident

USA) for frozen sectioning onto glass slides. Slides were dried at 37°C for 1 hour. After one rinse in PBS, sections were permeabilized for 5 minutes in 0.1% Triton X-100 and rinsed twice in PBS. Sections were blocked in 10% normal goat serum (for carbonic anhydrase II [CAII] antibody samples) or 10% normal donkey serum (for CAIV antibody samples) for 30 minutes before incubation in primary antibody for 2 hours at room temperature (rabbit anti-CAII, 1:100, sc-25596; Santa Cruz Biotechnology, Inc.) or 37°C (goat anti-CAIV, 1:100, sc-17247; Santa Cruz Biotechnology, Inc.). After three additional washes in PBS, sections were incubated 1 hour at room temperature with fluorescently conjugated secondary antibody (goat anti-rabbit IgG-Alexa 568, 1:1000; Invitrogen, Grand Island, NY, USA; donkey anti-goat IgG-FITC, 1:100, Santa Cruz Biotechnology, Inc.). Samples were washed in PBS three times, then mounted in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). Images were acquired by epifluorescence microscopy (Zeiss Axio Observer; Carl Zeiss, Jena, Germany).

Histochemical Stain for Carbonic Anhydrase Activity

Tissues for histochemical staining of carbonic anhydrase activity were prepared as follows. Human corneal tissue was incubated in either Ringer's solution with addition of 100 μM of a CAI (brinzolamide or dorzolamide) or vehicle (dimethyl sulfoxide [DMSO] or water) for 30 minutes at 37°C. Tissues were then fixed in 3% paraformaldehyde for 30 minutes, rinsed in PBS twice and then mounted in OCT embedding medium for frozen sectioning.

Histochemical stain for carbonic anhydrase activity in human corneal tissue samples was performed according to published techniques with modifications as described here.[14,15] Frozen sections (12-μm thickness) were cut on a cryostat, immediately transferred flat onto a thin support mesh (matte tulle, 100% nylon; Jo-Ann Fabric and Craft Stores, Hudson, OH, USA), transferred to microfuge tubes, and kept frozen (on dry ice for transport and at –80°C in the laboratory) until use. Solutions for Hansson's stain were prepared fresh immediately before use. A cobalt solution was prepared by combining 5.0 mL 0.2 M $CoSO_4$, 30.0 mL 0.53 M H_2SO_4, 15.0 mL 67 mM KH_2PO_4, and 30 mL H_2O. Sodium bicarbonate (0.38 g) was dissolved in 20 mL H_2O in a 50-mL Erlenmeyer flask with moderate stirring. Once dissolved, 8.5 mL of cobalt solution was added to the flask, mixed with moderate stirring for 4 minutes, and allowed to stand for 4 minutes. This reaction mix was then immediately transferred to wells in a 6-well plate (10 mL/well). A CAI (either 100 μM brinzolamide or 100 μM dorzolamide) or control (water or DMSO) was added to each well. A circle of thin nylon support mesh the size of the well was overlaid on the surface of the reaction mixture and the mesh with the frozen tissue section was placed tissue-side down on top of that immediately on removal from storage at –80°C. The tissue released from its original support and floated on the surface of the reaction mix above the larger circle of mesh in the well. This support was used to transfer the tissue between solutions until the end of the reaction and washes. The tissue was incubated in reaction mix for 2 minutes followed by a 1-minute wash in 10% sucrose. Samples were then incubated for 2 minutes in 2% ammonium sulfide and washed three times in 10% sucrose for 1 minute each. Samples were mounted on microscope slides with Vectashield mounting medium with DAPI (Vector Laboratories, Inc.). Specimens were examined by microscopy for deposition of black precipitate in regions of carbonic anhydrase activity.

Results

Characteristics of Human Donor Corneal Tissue

The optimal criteria for I_{sc} recordings from eye bank–stored human donor corneal tissue has not been established. From our initial recordings, we determined that the most stable recordings were obtained from tissue with a death-to-preservation time of less than 12 hours and when experiments were performed within 7 days postmortem. Longer time periods did not preclude good measurements, but quality of the results was more variable. Donor age or IOL status (phakic or pseudophakic) did not have any apparent influence. The most common reasons the tissues were not used for transplantation were positive blood cultures or corneal infiltrates. These small peripheral infiltrates did not affect the I_{sc} recordings. Characteristics of the human corneal tissues used for these experiments are summarized in Table 1.

To establish whether the corneal tissue storage solution was influencing the physiologic recordings, three pairs of corneas were selected with one cornea of each pair being maintained at 4°C in storage solution while the other cornea was cultured at 37°C for 4 to 8 days in a 5% CO_2 humidified incubator in culture medium (Opti-MEM I Medium [catalogue no. 31985-070; Invitrogen] + 10% fetal bovine serum + 1× antibiotic-antimycotic [Invitrogen] + 200 mg/L calcium chloride). Paired I_{sc} recordings were performed with cold-stored and cultured corneas. For one pair of corneas, culture increased the I_{sc} amplitude. For another, it decreased. For the final pair, there was no change (data not shown). Based on these results, culturing did not have a definitive benefit. Thus, all experiments on human tissue were performed with corneas equilibrated in Ringer's solution for approximately 15 minutes before mounting in the Ussing chambers.

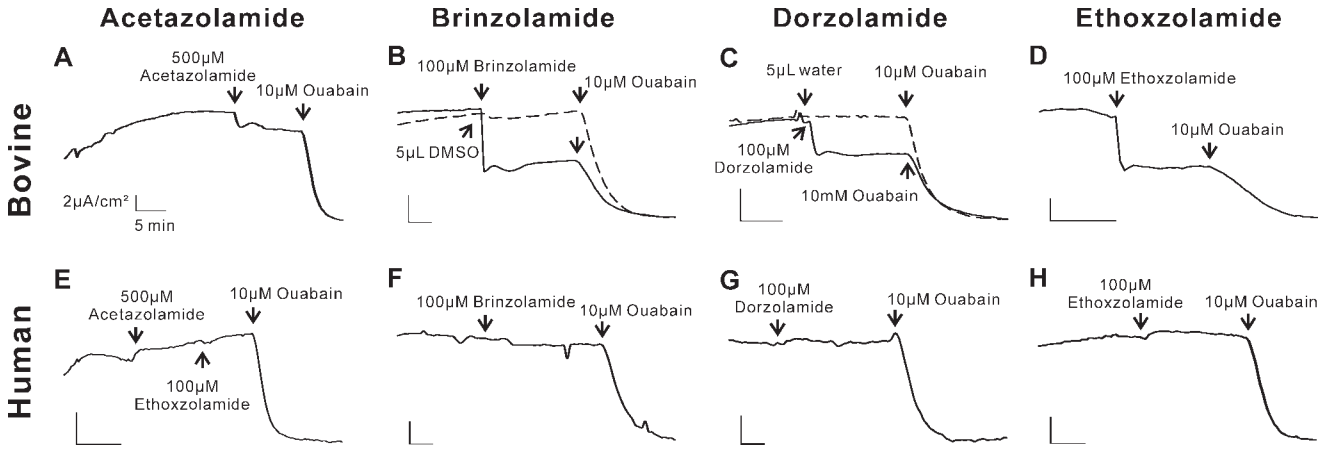

Figure 1. Effects of CAIs on bovine and human corneal endothelial short-circuit current. Representative short-circuit current traces from bovine (**A–D**) and human (**E–H**) corneal endothelium in response to the indicated drugs. *Dashed lines* in (**B**, **C**) represent concurrent control recordings with addition of vehicle without drug.

Carbonic Anhydrase Inhibitor Effect on Bovine and Human Corneal Endothelial I_{sc}

The I_{sc} for both bovine and human corneal endothelial recordings was noted to stabilize within 20 to 30 minutes of placement in the recording chambers. During this period, there was an increase in I_{sc} and a decrease in resistance followed by a plateau. Ouabain, a blocker of the Na^+/K^+ ATPase, which is central to maintaining gradients for Na^+ and K^+ and cell membrane potential, was added at the conclusion of recordings to determine the baseline (zero point) for calculation of the final total I_{sc}. At the I_{sc} plateau before experimental drug addition, the mean maximum I_{sc} for all experiments for this study combined was 14.1 ± 3.7 μA/cm² ($n = 48$) for bovine and 4.8 ± 3.0 μA/cm² ($n = 13$) for human. The transendothelial resistance at plateau before drug addition was 18.4 ± 3.0 Ω·cm² ($n = 48$) for bovine and 14.6 ± 4.6 Ω·cm² ($n = 13$) for human. The average resistance was minimally affected by drug addition.

Four CAIs were tested in both species: 500 μM acetazolamide, 100 μM brinzolamide, 100 μM dorzolamide, and 100 μM ethoxzolamide. These concentrations were selected based on concentrations used in prior published studies.[2,16,17] Representative I_{sc} recordings are shown in Figure 1. All CAIs produced a decrease in bovine corneal endothelial I_{sc} (% change in I_{sc}: acetazolamide, −21.0 ± 9.5, $n = 8$; brinzolamide, −35.5 ± 13.5, $n = 9$; dorzolamide, −33.6 ± 7.2, $n = 8$; ethoxzolamide, −35.3 ± 12.9, $n = 8$). Acetazolamide consistently had the least effect in decreasing I_{sc}. Brinzolamide, dorzolamide, and ethoxzolamide all produced a rapid decrease in I_{sc}. For all CAIs, the decrease in I_{sc} frequently produced a pattern of current overshoot followed by a sinusoidal equilibration. In contrast, no such rapid decrease in I_{sc} was noted for human corneal endothelium. Three of the four CAIs showed a nonsignificant trend toward an increase in I_{sc} (% change in I_{sc}: acetazolamide, 16.2 ± 20.1, $n = 3$; brinzolamide, 6.7 ± 13.9, $n = 3$; dorzolamide, 8.0 ± 20.4, $n = 3$; ethoxzolamide, −4.8 ± 10.3, $n = 2$). For all drugs, the differences in I_{sc} between bovine and human corneal tissue were significant (acetazolamide $P = 0.004$, brinzolamide $P = 0.0009$, dorzolamide $P = 0.0005$, and ethoxzolamide $P = 0.02$). Data are summarized in Table 2. Addition of vehicle (DMSO or water) without drug did not affect I_{sc} (Figs. 1B, 1C).

Table 2. Effect of CAIs on Corneal Endothelial Short-Circuit Current in Human and Bovine Tissue

	% Change in Corneal Endothelial I_{sc} (n)		
Drug	**Bovine**	**Human**	***P* Value**
Acetazolamide	−21.0 ± 9.5 (8)	16.2 ± 20.1 (3)	0.004
Brinzolamide	−35.5 ± 13.5 (9)	6.7 ± 13.9 (3)	0.0009
Dorzolamide	−33.6 ± 7.2 (8)	8.0 ± 20.4 (3)	0.0005
Ethoxzolamide	−35.3 ± 12.9 (8)	−4.8 ± 10.3 (2)	0.02

Wigham and Hodson[13,18] demonstrated in 1981 that I_{sc} is dependent on HCO_3^- in both bovine and human corneal endothelium. The species differences in CAI effect that we observed prompted us to consider the potential contribution of carbonic anhydrase in facilitating HCO_3^- transport. Bovine corneal endothelial I_{sc} was measured in bicarbonate-free Ringer's and monitored following the stepwise addition of $NaHCO_3$ with dorzolamide added before or after HCO_3^- addition (Fig. 2). With the addition of HCO_3^- to a final concentration of 3 mM, bovine I_{sc} was not different in the initial presence or absence of 100 μM dorzolamide (I_{sc} at plateau with 3 mM HCO_3^- as % of $I_{sc,max}$ with 12 mM HCO_3^-: with dorzolamide prior = 58.3% ± 6.4%, $n = 5$; without dorzolamide prior = 59.7% ± 5.0%, $n = 3$; $P = 0.77$). Despite the absence of bicarbonate and exogenous CO_2, initial application of 100 μM dorzolamide resulted in a decrease in I_{sc}. We speculate that effect may be secondary to endogenous CO_2 production. The dorzolamide effect was not seen in human corneal endothelium although a similar response was noted with increasing bicarbonate concentration (Fig. 2C). The prominent rapid transient decrease in I_{sc} with HCO_3^- addition was noted for all ($n = 3$) human corneal endothelial recordings. The magnitude varied, possibly related to the rate of mixing in the chamber or to the data acquisition time points (data points were obtained every 20 seconds, thus the peaks of rapid changes may have been missed). However, such transient changes were seen infrequently and with much smaller magnitude when observed in bovine recordings. This difference may reflect alternative HCO_3^- transport and equilibration dynamics between species.

Presence and Activity of Carbonic Anhydrase in Human Corneal Endothelium

Because no effects of CAIs were noted in human corneal endothelium, we questioned if carbonic anhydrase was present in the tissue. We investigated the presence of carbonic

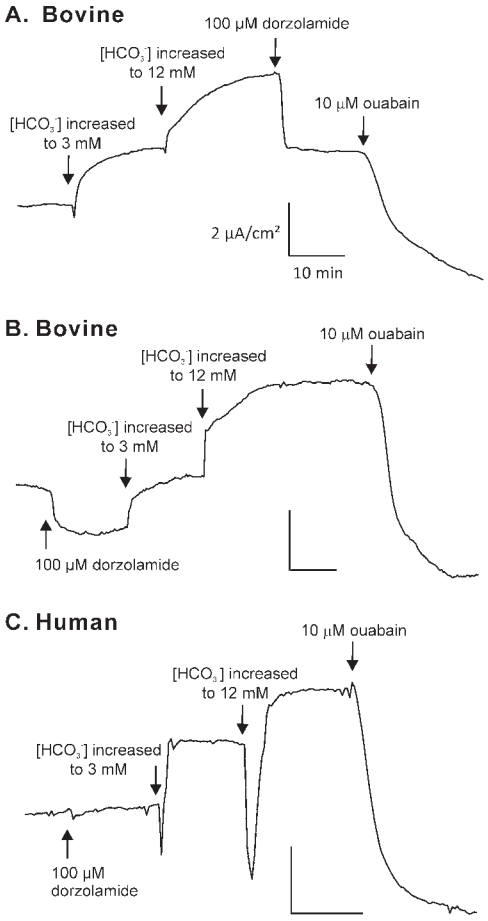

Figure 2. Bicarbonate and CAI (dorzolamide) effects on bovine and human corneal endothelial short-circuit current. Bovine corneas with stepwise increases in bicarbonate concentration from 0 mM (start) to 12 mM (end) with dorzolamide addition after (**A**) or before (**B**) bicarbonate additions. (**C**) Corresponding recording from human corneal endothelium.

anhydrase isoforms II and IV in human corneal endothelium by using specific antibodies for immunofluorescence localization. These experiments were performed on paired human corneas, with one cornea being used for I_{sc} measurement to confirm lack of CAI response (data shown in Fig. 1, brinzolamide and

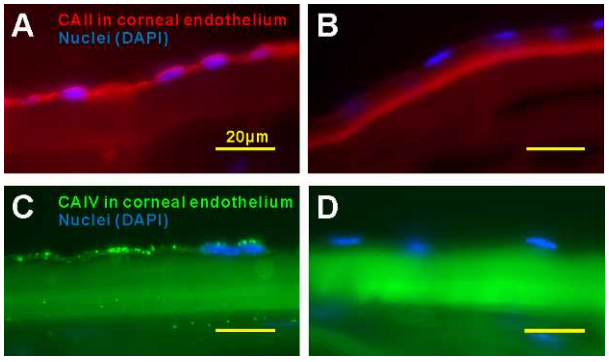

Figure 3. Immunolocalization of CAII and CAIV in human corneal endothelium. (**A**) CAII (*red*). (**C**) CAIV (*green*). (**B**, **D**) Negative controls (–) primary antibody, (+) secondary antibody with strong autofluorescence of Descemet's membrane. Nuclei (DAPI, *blue*). *Scale bars*: 20 μm.

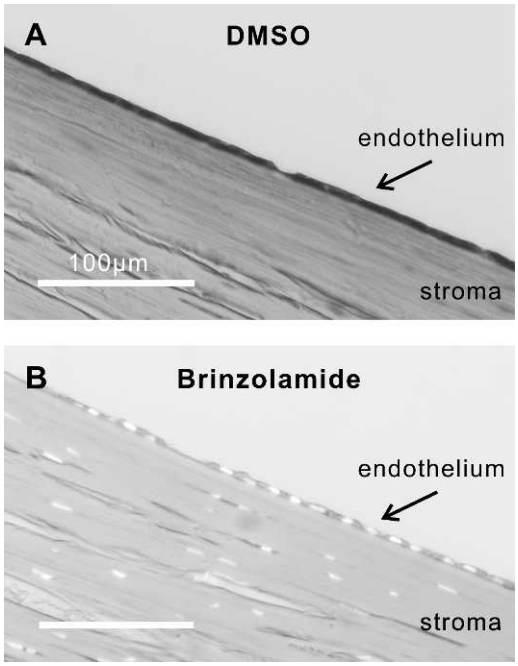

Figure 4. Carbonic anhydrase activity in human corneal endothelium by Hansson's histochemical staining. (**A**) Black precipitate in corneal endothelial cell monolayer indicates presence of carbonic anhydrase activity. (**B**) Absence of black precipitate in corneal endothelial cell monolayer when reaction is performed in the presence of 100 μM brinzolamide. In the absence of positive staining, the presence of corneal endothelial cells is confirmed with DAPI stain of the nuclei (*white*).

dorzolamide) and the other cornea of the pair being fixed for immunofluorescence localization studies. We found that both carbonic anhydrase isoforms II and IV were present in the human corneal endothelium (Figs. 3A, 3C). Control experiments without primary antibody showed no signal (Figs. 3B, 3D).

To demonstrate whether the carbonic anhydrase present in the human corneal endothelium was enzymatically active, we used the Hansson's histochemical staining procedure for carbonic anhydrase activity. Using the same human corneal tissue as used above for immunofluorescence localization, we found that the corneal endothelium had carbonic anhydrase activity, indicated by a black precipitate that was inhibited by addition of the CAI brinzolamide (100 μM) to the Hansson's stain reaction solution (Fig. 4).

Discussion

Our data reveal a distinct species difference in the corneal endothelial transport physiology of human versus bovine corneal endothelium. That human corneal endothelial physiology would be different from other species is not surprising. Several experiments from the 1970s and 1980s described the varied electrophysiologic responses of human, monkey, and rabbit corneas at baseline, in response to extracellular pH changes and in response to adrenergic modulation.[19–21] Following such descriptions, there was no further pursuit of the underlying mechanisms for these differences. In contrast, species differences in fluid transport of the ciliary epithelium have been appreciated and accounted for in experimental design.[22,23] Although fluid transport in rabbit ciliary epithelium is dependent on bicarbonate, in human, bovine, and porcine, it is dependent on chloride.

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Our data support prior observations that bovine and human corneal endothelial I_{sc} is carried mainly by HCO_3^-.[13,18] In addition, we show that CAI application does not prohibit an increase in I_{sc} in response to increasing HCO_3^- concentration in both species. Thus, more than half of the short-circuit current in both species is dependent on HCO_3^- and independent of carbonic anhydrase activity.

There are several potential explanations for why the HCO_3^- response may be independent of carbonic anhydrase activity and why carbonic anhydrase may be present in human corneal endothelium but without an effect on I_{sc}. Carbonic anhydrase activity facilitates the buffering of H^+ and HCO_3^-. Accordingly, ion transport altered by CAIs would involve H^+ and/or HCO_3^- pathways or pH-regulated processes. Several membrane transporters for H^+ and HCO_3^- (e.g., Na^+/H^+ exchanger; Na^+/HCO_3^- cotransporter; monocarboxylate transporters 1, 2, and 4) have been identified in corneal endothelium in animal species; however, data for humans are limited.[4,24–27] One possible explanation for lack of CAI effect in human corneal endothelium is lack of expression of membrane transporters necessary for using the benefit of increased buffering by carbonic anhydrase. A second possibility is that higher concentrations of CAIs are necessary to see an effect on human corneal endothelium. Another possibility is that, unlike rabbit and bovine, human corneal endothelial transport uses pathways independent of a need for buffering by carbonic anhydrase. Our bovine data, consistent with previously published data, demonstrate a one-third decrease in I_{sc} with CAIs, but two-thirds of the current remains.[28] We do not know the underlying cause of this current, but it may involve the same mechanism as the carbonic anhydrase-independent process of human corneal endothelium. The carbonic anhydrase–independent process may be independent of carbonic anhydrase catalytic activity but may still incorporate the ability of the molecule to shuttle protons to proton-dependent transporters, such as the monocarboxylate transporter.[29,30] This "proton-collecting antenna" model derived from astrocytes for CAII and monocarboxylate transporter 1 may also be present in corneal endothelium. It is appealing to consider that multiple ion transport mechanisms in the corneal endothelium may be contributing to fluid homeostasis. Such reasoning might explain why individuals with corneal endothelial guttata show greater tendency to swell with topical CAIs.[7] Although this could be due to a decrease in carbonic anhydrase activity, we speculate there may be upregulation of a carbonic anhydrase–dependent mechanism in compromised corneal endothelium. It is also possible that although an increase in corneal thickness is not noted with CAIs in noncompromised human corneas, a more sensitive measurement of changes in intrastromal pressure may reveal subtle differences.[31]

Human tissue research is challenging secondary to tissue availability, expense, and quality. However, our data highlight the importance of verifying observations from other species with human tissue experimentation if we are to make correlations and ultimately solve the problems of human eye disease. As demonstrated by these data, current eye-banking methodology for corneal tissue preservation extends our ability to use corneal tissue in a manner not possible several decades ago. Underlying genetic variability and potential systemic factors relating to the health of the donor may always confound the data analysis, but that variability is also within the nature of the human population we aim to understand and treat.

Acknowledgments

Supported by an Alcon Research Institute Young Investigator Grant (SPP); the Ralph Hochstetter Medical Research Fund in honor of Henry C. and Bertha H. Buswell (SPP); University at Buffalo, School of Medicine, Summer Research Fellowship (TMM); Research to Prevent Blindness unrestricted grant; and by facilities and resources provided by the Veterans Administration Western New York Healthcare System. The contents of this publication do not represent the views of the Department of Veterans Affairs or the US government.

Disclosure: **T.M. Malikowski**, None; **J.B. Bosch**, None; **S. Min**, None; **M.E. Duffey**, None; **S.P. Patel**, Alcon Research Institute (F)

References

1. Bonanno JA. Molecular mechanisms underlying the corneal endothelial pump. *Exp Eye Res*. 2012;95:2–7.
2. Fischbarg J, Lim JJ. Role of cations, anions and carbonic anhydrase in fluid transport across rabbit corneal endothelium. *J Physiol*. 1974;241:647–675.
3. Diecke FP, Wen Q, Sanchez JM, Kuang K, Fischbarg J. Immunocytochemical localization of Na^+-HCO_3^- cotransporters and carbonic anhydrase dependence of fluid transport in corneal endothelial cells. *Am J Physiol Cell Physiol*. 2004;286:C1434–C1442.
4. Liu C, Cheng Q, Nguyen T, Bonanno JA. Knockdown of NBCe1 in vivo compromises the corneal endothelial pump. *Invest Ophthalmol Vis Sci*. 2010;51:5190–5197.
5. Egan CA, Hodge DO, McLaren JW, Bourne WM. Effect of dorzolamide on corneal endothelial function in normal human eyes. *Invest Ophthalmol Vis Sci*. 1998;39:23–29.
6. Giasson CJ, Nguyen TQ, Boisjoly HM, Lesk MR, Amyot M, Charest M. Dorzolamide and corneal recovery from edema in patients with glaucoma or ocular hypertension. *Am J Ophthalmol*. 2000;129:144–150.
7. Wirtitsch MG, Findl O, Heinzl H, Drexler W. Effect of dorzolamide hydrochloride on central corneal thickness in humans with cornea guttata. *Arch Ophthalmol*. 2007;125:1345–1350.
8. Adamsons I. Irreversible corneal decompensation in patients treated with topical dorzolamide. *Am J Ophthalmol*. 1999;128:774–776.
9. Konowal A, Morrison JC, Brown SV, et al. Irreversible corneal decompensation in patients treated with topical dorzolamide. *Am J Ophthalmol*. 1999;127:403–406.
10. Tanimura H, Minamoto A, Narai A, Hirayama T, Suzuki M, Mishima HK. Corneal edema in glaucoma patients after the addition of brinzolamide 1% ophthalmic suspension. *Jpn J Ophthalmol*. 2005;49:332–333.
11. Zhao JC, Chen T. Brinzolamide induced reversible corneal decompensation. *Br J Ophthalmol*. 2005;89:389–390.
12. Wistrand PJ, Schenholm M, Lonnerholm G. Carbonic anhydrase isoenzymes CA I and CA II in the human eye. *Invest Ophthalmol Vis Sci*. 1986;27:419–428.
13. Wigham C, Hodson S. The effect of bicarbonate ion concentration on trans-endothelial short circuit current in ox corneas. *Curr Eye Res*. 1981;1:37–41.
14. Hansson HP. Histochemical demonstration of carbonic anhydrase activity. *Histochimie*. 1967;11:112–128.
15. Sugai N, Ito S. Carbonic anhydrase, ultrastructural localization in the mouse gastric mucosa and improvements in the technique. *J Histochem Cytochem*. 1980;28:511–525.
16. Bonanno JA, Srinivas SP, Brown M. Effect of acetazolamide on intracellular pH and bicarbonate transport in bovine corneal endothelium. *Exp Eye Res*. 1995;60:425–434.
17. Srinivas SP, Ong A, Zhai CB, Bonanno JA. Inhibition of carbonic anhydrase activity in cultured bovine corneal endothelial cells by dorzolamide. *Invest Ophthalmol Vis Sci*. 2002;43:3273–3278.
18. Wigham C, Hodson S. Bicarbonate and the trans-endothelial short circuit current of human cornea. *Curr Eye Res*. 1981;1:285–290.

19. Fischer F, Voigt G, Liegl O, Wiederholt M. Effect of pH on potential difference and short circuit current in the isolated human cornea. *Pflugers Arch*. 1974;349:119–131.
20. Fischer FH, Schmitz L, Hoff W, Schartl S, Liegl O, Wiederholt M. Sodium and chloride transport in the isolated human cornea. *Pflugers Arch*. 1978;373:179–188.
21. Wiederholt M, Schmidt DK, Eggebrecht R, Zimmermann J, Fischer FH. Adrenergic regulation of sodium and chloride transport in the isolated cornea of rabbit and man. *Graefes Arch Clin Exp Ophthalmol*. 1983;220:240–244.
22. Candia OA, To CH, Gerometta RM, Zamudio AC. Spontaneous fluid transport across isolated rabbit and bovine ciliary body preparations. *Invest Ophthalmol Vis Sci*. 2005;46:939–947.
23. Candia OA, To CH, Law CS. Fluid transport across the isolated porcine ciliary epithelium. *Invest Ophthalmol Vis Sci*. 2007; 48:321–327.
24. Chidlow G, Wood JP, Graham M, Osborne NN. Expression of monocarboxylate transporters in rat ocular tissues. *Am J Physiol Cell Physiol*. 2005;288:C416–428.
25. Nguyen TT, Bonanno JA. Bicarbonate, NBCe1, NHE, and carbonic anhydrase activity enhance lactate-H^+ transport in bovine corneal endothelium. *Invest Ophthalmol Vis Sci*. 2011; 52:8086–8093.
26. Schmedt T, Chen Y, Nguyen TT, Li S, Bonanno JA, Jurkunas UV. Telomerase immortalization of human corneal endothelial cells yields functional hexagonal monolayers. *PloS One*. 2012; 7:e51427.
27. Sun XC, Bonanno JA. Identification and cloning of the Na/HCO_3^- cotransporter (NBC) in human corneal endothelium. *Exp Eye Res*. 2003;77:287–295.
28. Hodson S, Miller F. The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. *J Physiol*. 1976;263:563–577.
29. Becker HM, Klier M, Schuler C, McKenna R, Deitmer JW. Intramolecular proton shuttle supports not only catalytic but also noncatalytic function of carbonic anhydrase II. *Proc Natl Acad Sci U S A*. 2011;108:3071–3076.
30. Stridh MH, Alt MD, Wittmann S, et al. Lactate flux in astrocytes is enhanced by a non-catalytic action of carbonic anhydrase II. *J Physiol*. 2012;590:2333–2351.
31. Teus MA, Bolivar G, Alio JL, Lipshitz I. Short-term effect of topical dorzolamide hydrochloride on intrastromal corneal pressure in rabbit corneas in vivo. *Cornea*. 2009;28:206–210.

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