Inhibition of Carbonic Anhydrase Activity in Cultured Bovine Corneal Endothelial Cells by Dorzolamide

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PURPOSE. Fluid transport by the corneal endothelium is dependent on the presence of HCO₃⁻ and the activity of carbonic anhydrase (CA)-II and -IV, the cytoplasmic and membrane-bound CAs, respectively. This study was conducted to examine the inhibition of CA activity in cultured bovine corneal endothelial cells (BCECs) by dorzolamide, a topical CA inhibitor used in glaucoma therapy.

METHODS. BCECs were grown on glass coverslips and then perfused with HCO₃⁻-free Ringer’s. The inward flux of CO₂ was induced by exposure to CO₂/HCO₃⁻ Ringer’s and the opposing outward flux by returning to HCO₃⁻-free Ringer’s. Consequent transients in intracellular pH (pHi) were measured using the pH-sensitive fluorescent dye 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). During the inward flux of CO₂, the maximum rate of change of pH, was taken as a quantitative measure of the overall CA activity in BCECs.

RESULTS. Exposure to CO₂/HCO₃⁻ Ringer’s led to a transient decrease in pHi (component A), followed by a rapid increase to a new steady state (component B). However, when the CO₂/HCO₃⁻ Ringer’s was removed, the pH increased transiently (component C) and then rapidly returned to the original pHi (component D). Component A, caused by an inward flux of CO₂ and its subsequent hydration by CA-II, was blocked by dorzolamide in a dose-dependent manner with an 50% inhibitory concentration (IC₅₀) of 2.4 µM (95% confidence interval: 0.5–10.85 µM). However, the inhibition of the outward flux of CO₂, inward flux of HCO₃⁻, and outward flux of HCO₃⁻ (associated with components C, B, and D, respectively) was not dose dependent. Cells that were exposed to 500 nM of the drug for longer than 30 minutes did not show a significantly greater inhibition of any of the components. Dorzolamide and acetazolamide (500 µM) did not show additive inhibition of any of the components (P = 0.13; n = 6).

CONCLUSIONS. Dorzolamide significantly inhibits CA activity in BCECs at micromolar levels. Because these levels are encountered in the cornea and aqueous humor after topical administration, dorzolamide may compromise corneal hydration control, especially when the functional reserve of corneal endothelium is low. Dorzolamide does not appear to accumulate in the cells, because the inhibition of CA-II did not increase after prolonged exposure to the drug. (Invest Ophthalmol Vis Sci. 2002;43:3273–3278)

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Ocular approach to reducing intraocular pressure in the medical management of glaucoma is to decrease the rate of secretion of aqueous humor by inhibiting cytosolic carbonic anhydrase (CA-II isoenzyme) in the nonpigmented ciliary epithelium.1,2 Oral administration of the prototypical carbonic anhydrase (CA) inhibitor acetazolamide, which is known to have poor transcorneal permeability,3,4 is not well tolerated and occasionally causes systemic side effects.5,6 Dorzolamide and brinzolamide are currently being prescribed for topical administration.1,2,7 Dorzolamide is permeable across the cornea, is a selective inhibitor of the CA-II isoenzyme,3,8–10 and hence is found to be efficacious in reducing intraocular pressure with little or no risk of systemic side effects.1,3,11,12

Findings in several clinical studies involving both healthy volunteers and patients with glaucoma suggest that topical dorzolamide does not have any significant effects on fluid transport activity by the corneal endothelium,1,13–18 which is essential for corneal transparency. This apparent absence of effect is enigmatic, because fluid transport by the endothelium is driven by such HCO₃⁻-dependent ion transport mechanisms as the Na⁺/HCO₃⁻ cotransport (NBC1),19–20 Cl⁻/HCO₃⁻ exchange (AE2),21,22 and cAMP-dependent HCO₃⁻ conductance23 (Fig. 1). The HCO₃⁻ fluxes mediated by these mechanisms are influenced by transmembrane gradients and intracellular levels of CO₂ and HCO₃⁻ which are, in turn, determined by intracellular pH (pHi) and the activities of cytosolic CA-II24 and membrane-bound CA-IV isoenzymes.19,25,26 In addition, the HCO₃⁻ flux mediated by the electrogenic Na⁺/HCO₃⁻ cotransporter would be affected by the membrane potential at the basolateral membrane. Consistent with these findings, CA inhibitors are known to inhibit CO₂/HCO₃⁻-dependent ionic fluxes in cultured endothelial cells27,28 and fluid transport activity in rabbit corneas mounted in vitro.27,28 Therefore, the insignificant effect of topical dorzolamide in corneal thickness could be attributed to a low concentration of the drug in the aqueous humor and stroma and/or to the substantial functional reserve of the corneal endothelial cells attributed to excess cell density.29 Accordingly, corneas with a compromised endothelial layer (such as in the elderly or patients with Fuchs’ dystrophy, corneal transplants, and cataract surgery) can irreversibly compensate after topical administration of dorzolamide.30 In this study, we characterized the effect of dorzolamide on the inhibition of CA activity in cultured bovine corneal endothelial cells (BCECs) at the concentrations encountered in the stroma and aqueous humor after topical administration. The results are useful for assessing the potential side effects of topical dorzolamide in patients with either a compromised endothelium or reduced aqueous clearance.

MATERIALS AND METHODS

Cell Culture

Primary cultures from fresh cow eyes were established in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and antibiotic-antimycotic (consisting of 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B), gassed at 37°C with 5% CO₂ and 95% air, and fed every 2 to 3 days, as described...
Experimental Protocol

After the perfusion chamber was mounted on the microscope, the cells were perfused with HCO₃⁻-free Ringer’s until they reached a steady state pHᵢ (i.e., fluorescence ratio of F490/F440). Cells were exposed to CO₂-HCO₃⁻ Ringer’s for 4 minutes and then returned to HCO₃⁻-free Ringer’s. Cells were exposed to dorzolamide once the pHᵢ response stabilized. After 3 minutes of equilibration in HCO₃⁻-free Ringer’s, cells were exposed for 4 minutes to CO₂-HCO₃⁻ Ringer’s that contained dorzolamide. The fluorescence ratio profile was later analyzed to calculate its first-time derivative by using the Savitzky-Golay convolution algorithm (Table 2D Software; SPSS Sciences, Chicago, IL) to locate the inflection points (i.e., the points at which the rate of change is maximum).³¹,³² The window size for smoothing required by the algorithm was set at 3%. The maximum slopes during the transients after perturbations were used to calculate the rates of inward and outward fluxes of CO₂ and HCO₃⁻ semiquantitatively (discussed later). In some experiments (shown in Fig. 4 for dorzolamide concentration = 500 nM), F490/F440 was recorded at 10 ratios/sec and was not included in the dose-response analysis (shown in Fig. 5).

Chlorides

BCECF-AM (catalog no. B-1170) was obtained from Molecular Probes (Eugene, OR). Dorzolamide was donated by Merck (Rahway, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

Statistics

Quantitative results are expressed as the mean ± SD. Paired t-tests (i.e., data from the same cells) were used for tests of significance to determine the additive effects of acetazolamide and dorzolamide on the overall CA activity in BCECs. The IC₅₀ (defined as the concentration at which the overall CA activity is inhibited by 50%) was calculated by fitting a hyperbolic curve, by using a nonlinear regression computer program (Prism; GraphPad, San Diego, CA).

RESULTS

We observed a characteristic pHᵢ response when cells perfused with HCO₃⁻-free Ringer’s were exposed to CO₂-HCO₃⁻-free Ringer’s (Fig. 2). The response consists of a transient acidification (component A) followed by a precipitous alkalization (component B) to a new steady state. The pHᵢ at the new steady state was much higher than that found in the absence of CO₂-HCO₃⁻. The extent of the increase in pHᵢ, as noted in our previous study,²² varies because it is dependent on multiple factors including membrane potential and transmembrane gradients of Na⁺ and HCO₃⁻. After cells were returned to HCO₃⁻-free Ringer’s, the pHᵢ returned to its original level (component D) but only after a transient alkalization (component C).

Next, the cells were exposed to dorzolamide (100 μM) dissolved in CO₂-HCO₃⁻-free Ringer’s. This led to a small acidification (see inset in Fig. 2), which vanished when the Ringer’s was completely free of trace levels of CO₂ (data not shown; examined in the context of acetazolamidic earlier).³³,³⁴ Finally, the same cells were exposed to CO₂-HCO₃⁻-free Ringer’s (containing dorzolamide at 100 μM) for a brief period, the pattern of pHᵢ responses was similar to the pattern created in absence of the drug. We then assessed the effect of dorzolamide on the rate of change of pHᵢ during the transients by calculating the percentage of the maximum slope of pHᵢ, i.e., the fluorescence ratio given by F490/F440 in the presence of dorzolamide compared with that in the absence of the drug for all components A, B, C, and D.

Although the general pattern of pHᵢ responses did not appear to be affected (Fig 2), the rates of change of pHᵢ for the

Measurement of pHᵢ

pHᵢ was measured as described previously,²¹–²⁵ with the use of the well-established pH-sensitive fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCEF). The cells were loaded with BCECF by exposing them to BCECF-acteyloxymethyl ester (AM) for 30 to 40 minutes²¹–²⁵ and then washing them for at least 30 minutes. Coverslips were then placed in a perfusion chamber maintained at 37°C and mounted on the stage of an inverted microscope. The Ringer’s solutions, also maintained at 37°C, were channeled into the perfusion chamber through an eight-way valve. The ratio of fluorescence emission (530 ± 10 nm), resulting from excitation of BCECF at 490 ± 5 and 440 ± 5 nm (denoted by F490/F440), was measured periodically (1-10 Hz). A photon-counting photomultiplier tube detected the emission intensity. The ratio F490/F440 is a linear function of pHᵢ,²¹–²⁵
different components (i.e., A, B, C, and D) were significantly reduced, indicating decreased fluxes of CO₂ and HCO₃⁻ in the presence of dorzolamide. Thus, component A, characterized by a transient decrease in pH, disappeared completely and component C, involving rapid alkalinization, was significantly reduced. The extent of inhibition of components A, B, C, and D from similar independent experiments for different concentrations of dorzolamide is summarized in Figure 3. The inhibition of component A was dose dependent, with the maximum amount of inhibition occurring at all concentrations exceeding 10 μM. A nonlinear regression (sigmoidal dose-response curve) of the extent of inhibition versus the concentration of dorzolamide indicated that the concentration of the drug required for IC₅₀ was 2.37 μM (95% confidence interval 0.51–10.85 μM). However, the other components showed responses that were not strictly dependent on the concentration of dorzolamide.

In comparison with acetazolamide, dorzolamide has a higher octanol–water partition coefficient. Therefore, dorzolamide is relatively more lipid soluble and penetrates the corneal epithelium when administered topically. Combined with higher lipophilicity, the two CA inhibitors are likely to differ in their apparent inhibition of the CA in the endothelium. This hypothesis was tested by determining the effect of both drugs together on the CO₂-HCO₃⁻ fluxes. In the first series of experiments, cells were first exposed to acetazolamide (500 μM) and then to dorzolamide in the presence of acetazolamide. In the second series of experiments, cells were

FIGURE 2. Reduced CO₂-HCO₃⁻ fluxes in the presence of dorzolamide. Cells loaded with pH-sensitive fluorescent dye BCECF were exposed to CO₂-HCO₃⁻ Ringer’s solution. After reaching a steady state in pH, the cells were returned to CO₂-HCO₃⁻ free Ringer’s. The addition and removal of CO₂-HCO₃⁻ was repeated in the presence of dorzolamide (100 μM). The y-axis shows the ratio of fluorescence emission at excitation at 495 nm to that at 440 nm as a relative measure of pH changes. Component A: transient acidification, component B: sustained alkalinization, component C: transient alkalinization, and component D: sustained acidification. Inset: small acidification that appeared when the cells were exposed to dorzolamide (100 μM) dissolved in nominally CO₂-HCO₃⁻ ‘free’ Ringer’s. This acidification vanished, as noted in a previous study, when the Ringer’s was completely free of trace levels of CO₂.

FIGURE 3. Reduced (A, C) CO₂ and (B, D) HCO₃⁻ fluxes in the presence of dorzolamide compared with fluxes in the absence of the drug for each component (A–D, respectively). The y-axis for each plot is percentage of inhibition (i.e., 100 × [slope in the presence of dorzolamide/slope in the absence of dorzolamide]). Slopes were calculated at the inflection points of the associated pH transients, as described in Figure 2 (also see Fig. 4) but obtained at different concentrations of dorzolamide. The inflection points were determined by the Savitzky-Golay algorithm. Numbers above each error bar (±SD) represent number of experiments, with separate coverslips.
first exposed to dorzolamide and then to acetazolamide in the presence of dorzolamide. A typical experimental profile is shown in Figure 5. The maximum slopes of the components A, B, C, and D in both protocols were compared. The presence of dorzolamide significantly affected the slopes at the inflection points corresponding to component C with and without dorzolamide. Peak changes in pH are a1, a2, a3, a4, and a5. Similarly, c1, c2, c3, c4, and c5 are the inflection points corresponding to component C with and without dorzolamide. Peak changes in pH are identified as c1, c2, c3, c4, and c5. $\frac{d\text{pH}}{dt}$ represents the slope of pH versus time profile obtained by the Savitzky-Golay algorithm.31,32 The top and bottom curves are associated with the left and right y-axes, respectively, as indicated by horizontal arrows.

**FIGURE 4.** Extent of inhibition of CO$_2$-HCO$_3^-$ fluxes remained unaltered during exposure to dorzolamide for a longer period. Cells loaded with BCECF were exposed to CO$_2$-HCO$_3^-$ Ringer’s. After reaching a steady state, the cells were returned to CO$_2$-HCO$_3^-$ -free Ringer’s. This addition and removal of CO$_2$-HCO$_3^-$ was repeated three times consecutively in the presence of dorzolamide (500 nM). The results show that the presence of dorzolamide significantly affected the slopes at the inflection points for the different components (n = 6; data not shown; P = 0.13).

**DISCUSSION**

Immunohistochemical and physiological experiments both demonstrate expression of the CA-II and -IV isoenzymes in the corneal endothelium.24,25 The importance of CO$_2$ and HCO$_3^-$ in endothelial fluid transport and corneal transparency is evident by the presence of constitutively active ion transport mechanisms at the basolateral and apical domains of the endothelial cells (Fig. 1). The specific goal of this study was to examine the effects of dorzolamide, a topical CA inhibitor, on CO$_2$ and HCO$_3^-$ transport in the endothelium at a cellular level. The results show marked inhibition of CO$_2$ fluxes across the plasma membrane of the corneal endothelium by the drug.

**Inhibition of CO$_2$-HCO$_3^-$ Fluxes**

The experimental protocol to assess how CO$_2$-HCO$_3^-$ fluxes are affected by the presence of dorzolamide is similar to that used in a previous study in which the effects of acetazolamide were examined.22 Specifically, the previous study showed that the rate of change of pH is a semiquantitative measure of CO$_2$-HCO$_3^-$ fluxes across the plasma membrane. Thus, the rate of change of pH in components A, B, C, or D was calculated as the initial slope of the pH profile immediately after a perturbation. To avoid the bias inherent in a manual estimation of the initial slopes, we chose to determine the continuous first derivative of the measured pH trace in a given experiment by using the Savitzky-Golay algorithm. This choice facilitated rapid computer-aided calculation of the slopes at all points along the pH trace after a moving-average smoothing of the raw data. Because the inflection points form perfect landmarks as peaks on the first-derivative curve (see Fig. 4), they can be easily located without bias.51,52 In addition, the inhibitory effects of dorzolamide would be most sensitive at the inflection points. Thus, the inhibition by dorzolamide of various components (i.e., A, B, C, and D) could be easily calculated (Fig. 3). The results in Figure 3 clearly show that dorzolamide reduced the slope at the inflection point for all components and therefore suggest that the transmembrane CO$_2$-HCO$_3^-$ fluxes are inhibited in the presence of the drug. This finding is similar to that noted for acetazolamide in the previous study.22 However, what is more important is that dorzolamide’s inhibitory effects are significant at concentrations encountered after topical administration of the drug in humans. Schmitz et al.53 have estimated that the peak concentration of dorzolamide is 2.7 $\mu$M in the aqueous humor 4 to 6 hours after topical administration. Similarly, measurements and estimates of the concentration of dorzolamide in the corneal stroma have ranged from 20 to 100 $\mu$M.2,3,8,9,10 Specifically, it must be noted that the IC$_{50}$ calculated in the current study for a representative component of flux is in the range of concentration in the aqueous humor, but many times lower than that reported in the corneal stroma.

**Dose Dependency of the Apparent Inhibition of CO$_2$-HCO$_3^-$ Fluxes**

Component A was inhibited by dorzolamide in a dose-dependent manner (Fig. 3A). This reflects inhibition of facilitative transmembrane transport of CO$_2$. That the derivative of the pH graph with respect to time ($d\text{pH}/dt$) at the inflection point corresponding to component A is a semiqualitative indicator of this influx of CO$_2$ can be described as follows: The acidification during component A involves the rapid diffusion of CO$_2$ into cytosol and its subsequent hydration aided by CA into H$_2$CO$_3$ (CO$_2$+H$_2$O $\rightarrow$ H$_2$CO$_3$). The latter dissociates into HCO$_3^-$ and H$^+$ (i.e., H$_2$CO$_3$ $\rightarrow$ H$^+$ + HCO$_3^-$), which rapidly leads to intracellular acidification. Thus, the generation rate of H$^+$ and the corresponding acidification rate are dependent on the rate of influx of CO$_2$, rate of CO$_2$ hydration, and intrinsic buffering.
capacity in the cells. Slowing the rate of hydration with carbonic anhydrase inhibitors leads to quick accumulation of CO₂ within the cell, thereby reducing the driving force for influx of CO₂.

Component C, contributed by CO₂ efflux, was not inhibited in a strictly dose-dependent manner (Fig. 3C), although significant inhibition was found at small concentrations of the drug (i.e., 500 nM). Similar to component C, inhibitions of components B and D were caused by the electrogenic influx and efflux, respectively, of HCO₃⁻ through the Na⁺-HCO₃⁻ cotransporter at the basolateral membrane (Fig 1). The contribution of the Cl⁻-HCO₃⁻ exchanger, expressed in fresh endothelium, toward HCO₃⁻ influx and efflux is small in cultured BCECs, as demonstrated in a previous study by small changes in [Cl⁻] i in response to exposure to HCO₃⁻ or its removal. 35 An analysis similar to that of component A (preceding paragraph) of our claim that the dPH/dt is an indicator of CO₂-HCO₃⁻ fluxes during components B, C, and D is beyond the scope of this study (see Sohma et al. 36 for additional details on modeling transmembrane CO₂-HCO₃⁻ transport). However, we recall that our previous study, which examined initial Na⁺ fluxes for components B and D in the presence and absence of acetazolamide, indicated that net fluxes of Na⁺ were unaffected by similar maneuvers. Thus, dorzolamide does not inhibit the transporter. What we observed is the slowing of the subsequent hydration-dehydation of CO₂. That there was no strict dose dependency, also noted in a previous study, is mostly likely attributable to background uncatalyzed hydration-dehydation of CO₂.

**Apparent Absence of Effect on Endothelium**

As shown in Figure 4, the extent of inhibition did not change significantly, even after exposure to dorzolamide for longer periods. However, the initial exposure to CO₂-HCO₃⁻- Ringer's, happening minutes after exposure to the drug, inhibited the flux significantly. This clearly indicates a rapid transmembrane influx of dorzolamide. Further, the constancy of inhibition suggests that the amount of CA-II in the endothelial cells is not as large as the amount in red blood cells (RBCs), given that there was no further inhibition in the endothelial cells, even after 30 to 40 minutes of exposure. RBCs, on the other hand, accumulate up to 20 to 25 μM dorzolamide against a plasma concentration of approximately 0.054 μM. 5

Given the small amount of CA in the corneal endothelium and its importance in fluid transport, the apparent absence of effect of dorzolamide on corneal hydration noted in clinical studies could be attributed to the high rate of clearance of dorzolamide from the aqueous humor and corneal stroma. 3,6,10,9,34 Because dorzolamide is rapidly lost from the anterior chamber and corneal stroma, clinical parameters such as corneal thickness and endothelial shape or size are likely to be affected only for short periods. In fact, a recent pharmacokinetic study has reported the half-life of absorption and elimination in the anterior chamber to be 1.2 and 5.0 hours, respectively. These considerations lead us to conclude that the effect of dorzolamide would be significant only in patients with partially decompensated corneas.

**References**


