Bicarbonate, NBCe1, NHE, and Carbonic Anhydrase Activity Enhance Lactate-\(H^+\) Transport in Bovine Corneal Endothelium

Tracy T. Nguyen and Joseph A. Bonanno

**Purpose.** To identify and localize the monocarboxylate transporters (MCTs) expressed in bovine corneal endothelial cells (BCEC) and to test the hypothesis that buffering contributed by HCO\(_3^-\), sodium bicarbonate cotransporter (NBCe1), sodium hydrogen exchanger (NHE), and carbonic anhydrase (CA) activity facilitates lactate flux.

**Methods.** MCT1–4 expression was screened by RT-PCR, Western blot analysis, and immunofluorescence. Endogenous lactate efflux and/or change in pH were measured in BCEC in HCO\(_3^-\)-free or HCO\(_3^-\)-rich Ringer, with and without niflumic acid (MCT inhibitor), acetazolamide (ACTZ, a CA inhibitor), 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (Na\(^+\)/H\(^+\) exchange blocker), disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS; anion transport inhibitor), or with NBCe1-specific small interfering (si) RNA-treated cells.

**Results.** MCT1, 2, and 4 are expressed in BCEC. MCT1 was localized to the lateral membrane, MCT2 was lateral and apical, while MCT4 was apical. pH measurements showed significant lactate-induced cell acidification (LIA) in response to 20-second pulses of lactate. Incubation with niflumic acid significantly reduced the rate of pH change (dPH/dt) and lactate-induced cell acidification. EIPA inhibited alkalinization after lactate removal. Lactate-dependent proton flux was significantly greater in the presence of HCO\(_3^-\) but was reduced by ACTZ. Efflux of endogenously produced lactate was significantly faster in the presence of HCO\(_3^-\), was greater on the apical surface, was reduced on the apical side by ACTZ, as well as on the apical and basolateral side by NBCe1-specific siRNA, DIDS, or EIPA.

**Conclusions.** MCT1, 2, and 4 are expressed in BCEC on both the apical and basolateral membrane (BL) surfaces consistent with niflumic acid-sensitive lactate-H\(^+\) transport. Lactate-dependent proton flux can activate Na\(^+\)/H\(^+\) exchange and be facilitated by maximizing intracellular buffering capacity through the presence of HCO\(_3^-\). HCO\(_3^-\) transport, NHE and CA activity. (*Invest Ophthalmol Vis Sci.* 2011;52:8086–8093) DOI:10.1167/iovs.11-8086

**Corneal transparency** is a cumulative result of avascularity, regular arrangement of stromal collagen fibrils, maintenance of hydration by the corneal endothelium, and uniform refractive index within the epithelium aided by the relative lack of mitochondria. As a consequence of the paucity of epithelial mitochondria, the cornea is highly glycolytic. Eighty-five percent of glucose consumed by the cornea is converted to lactate, which results in a substantial [lactate] gradient between the cornea and aqueous humor. Accumulation of lactate, for example induced by hypoxia, creates an osmotic load that leads to corneal edema. Because the outer layers of corneal epithelium are impermeable to lactate, lactate efflux must occur posteriorly across the corneal endothelium and into the anterior chamber. Failure to remove lactate, even under normoxic conditions, will lead to corneal edema and loss of corneal transparency, indicating that lactate needs to be efficiently removed from the cornea.

Lactic acid has a pK\(_a\) of 3.86, so at physiological pH, 99.96% is the anion lactate − so cellular transport will need to be facilitated. Lactate produced in the epithelium is transported into the stroma via a lactate-H\(^+\) cotransport mechanism. Lactate efflux across the corneal endothelium could be transcellular or paracellular; however, because most of the surface area is cellular, it would be most efficient if stroma to aqueous efflux were transcellular. Indeed, facilitated cotransport of lactate-H\(^+\) has been demonstrated in the corneal endothelium. Using rabbit corneas, Giasson and Bonanno found that stromal lactate acidifies endothelial cells suggesting an H\(^+\) coupled mechanism. Since then, the monocarboxylate transporter family (MCT, SLC16) has emerged as the molecular mechanism for facilitated lactate-H\(^+\) cotransport.

MCTs are responsible for transport of lactate, pyruvate, and ketone bodies across the plasma membrane in many tissues. Of the fourteen members of the MCT family, only the first four members (MCT1–4) have been demonstrated experimentally to facilitate the proton-linked transport of lactate. MCT1 is the most widely distributed isofrom and is expressed in most mammalian tissues. MCT2 can be found in the liver, kidney, brain, and sperm tail. Although MCT1 and MCT2 can be found coexpressed in the same tissue, their locations within the tissue can be different, suggesting that each has a unique functional role. MCT3 has been found exclusively in the basolateral membrane (BL) of the retinal pigment epithelium (RPE) and choroid plexus epithelium. Lastly, MCT4 can be found in glycolytic tissues such as white skeletal muscle fibers, astrocytes, white blood cells, and chondrocytes. Chidlow et al. have examined the distribution of the MCT1–4 isoforms in the anterior structures of the rat eye, including the cornea. They found all four MCT mRNAs present in the rat cornea. Immunohistochemistry data however, showed immunoreactivity only to MCT1 and MCT2 in the corneal endothelium.

MCT dependent lactate-H\(^+\) flux is facilitated by bicarbonate transporters and carbonic anhydrase (CA) activity in various cells and tissues. Becker et al. reported that lactate transport activity by MCT1 is increased two-fold when coexpressed with the sodium bicarbonate cotransporter (NBC) in...
Xenopus oocytes. They also reported that MCT1 and MCT4 transport activity is increased by interaction with carbonic anhydrase II (CAII).10,14,15 Similarly, CA activity has been found to facilitate lactic acid transport in rat skeletal muscle fibers11 as well as in neurons and astrocytes.12 These studies suggest that HCO3− in conjunction with CA increases the effective buffering capacity and thus attenuates the dissipation of H+ gradient across the cell membrane hence increasing MCT activity.13–15 Buffering capacity has a marked influence on the change in pHi and is itself enhanced by the activity of many acid and/or base transporters in a cell. Because of the presence of HCO3− transporters, several CAs, and lactate-H+ cotransporters, we postulate that a similar facilitation mechanism occurs in the corneal endothelium. Interestingly, Giasson and Bonanno4 found that basolateral lactate influx in the rabbit corneal endothelium is partially sodium dependent suggesting that the basolateral sodium bicarbonate cotransporter (NBCe1) could act to augment lactate-H+ flux. There is also evidence that Na+/H+ exchange (NHE1, SLC9A1) exists in the corneal endothelium and is active when pHi is reduced.17 Lactate-induced acidification could activate NHE1, which may also be involved in regulating the transport of lactate-H+ across the corneal endothelium.

In this study, we have attempted to examine the role of buffering capacity in the transport of lactic acid in bovine corneal endothelium. We first sought to identify the MCT isoforms that facilitate lactate-H+ transport in cultured bovine CE and to determine their membrane localization. We then examined the effect of HCO3− and CA activity on buffering capacity and lactate-induced cell acidification (LIA). Direct lactate flux was examined by measuring endogenous lactate appearance in the apical and BL domains while inhibiting NBCe1, NHE1, and CA activity.

**Materials and Methods**

**Cell Culture**

Fresh bovine eyes were obtained from a local slaughterhouse and corneal endothelial cells were harvested as previously described.15 Primary culture was established in T-25 flasks with 5 mL Dulbecco modified Eagle medium (DMEM), 10% bovine calf serum (BCS) and 1% modified Eagle medium (DMEM), 10% bovine calf serum (BCS) and 1% antibiotics/antimycotic (penicillin 100 U/mL, and fungizone 0.25 μg/mL), gassed with 5% CO2-95% air at 37°C and medium changed every 2 to 3 days. Primary cultures were subcultured to T-25 flasks and grown to confluence, which was then used to further subculture onto 25-mm round glass coverslips, 12 mm 0.4 μm permeable polyester inserts (Transwell, Corning Life Sciences, cat.# 3460), or Nunc 10mm 0.2 μm tissue culture inserts (Anopore; Whatman cat# 136935) and grown to confluence within 3 to 5 days. The amount of BCS in the DMEM was reduced to 2% 24 hours before all physiological experiments.

**RNA Isolation and RT-PCR**

Total RNA was isolated from bovine RPE and corneal endothelium (RNeasy Kit; Qiagen, Valencia, CA). Reverse transcription was carried out (High Capacity RNA-to-cDNA Kit; Applied Biosystems) with 200 ng RNA in a 20 μL reaction. PCR was performed following manufacturer’s protocol (AmpliTag 360 DNA Polymerase Protocol; Applied Biosystems, Rockville, MD) using primers specific to the MCT-1-4 and β-actin. The nucleotide sequences of those primers are listed in Table 1. cDNA (1 μL) was added into a 25 μL PCR reaction that underwent 35-cycle amplification. PCR products were examined on 1.2% agarose gels stained with ethidium bromide. Negative control samples were cDNAs not subjected to reverse transcriptase. Bovine RPE was used as a positive control for MCT1-4 genes based on a previous report that showed all four MCT mRNAs present in the rat RPE. All primer oligonucleotides were obtained from Sigma Aldrich (St. Louis, MO).

**Immunoblotting**

Cultured bovine corneal endothelial cells (BCEC) were washed three times with chilled bicarbonate-free (BF) Ringer solution and then lysed (50 mM Tris base, 150 mM NaCl, 0.5% deoxycholic acid-sodium salt, 2% SDS and 1% NP40, pH 7.5, protease inhibitor cocktail) and sonicated (Branson 250, Danbury, CT) on ice. This was followed by centrifugation at 14,000 rpm for 15 minutes to pellet cell debris. The supernatant was collected and an aliquot was taken for protein concentration measurement (Pierce BCA Protein Assay; Thermo Scientific, Rockford, IL). 1X Laemmli sample buffer was added to 30 μg of protein samples and the mixture was heated in a 95°C heating block for 5 minutes to denature the protein. Samples were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Blots were then probed with MCT1, -2, -3, or -4 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA: sc-50325, sc-50323, sc-14930 and sc-50329, respectively; 1:1000 dilution) for 1 hour at room temperature. Secondary horseradish peroxidase conjugated antibody incubation was done for 1 hour at room temperature. Secondary horseradish peroxidase conjugated antibody incubation was done for 1 hour at room temperature. Secondary horseradish peroxidase conjugated antibody incubation was done for 1 hour at room temperature. Blots were then probed with MCT1-4 genes based on a previous report that showed all four MCT mRNAs present in the rat RPE. All primer oligonucleotides were obtained from Sigma Aldrich (St. Louis, MO).

**Immunofluorescence**

Immunofluorescence staining was performed on BCEC cultured on 25 mm coverslips as previously described.16 Rabbit anti-MCT1, MCT3, and MCT4 polyclonal antibodies were diluted 1:50 in equal mixture of PBS-goat serum and chicken anti-MCT2 polyclonal antibody was diluted 1:100. Rat anti-ZO-1 (1:30) monoclonal antibody was added to the mixture of each MCT for 1 hour at room temperature. Coverslips were washed three times for 15 minutes in PBS containing 0.1% Triton X-100.

**Table 1. PCR Primer Sequences Used to Determine the Expression of Bovine Monocarboxylic Acid Transporter Genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Sequences</th>
<th>Positions (at cDNA)</th>
<th>PCR Prod. Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT1</td>
<td>Forward 5’-GGAGTCATTTGGAGGCTTGGG-3’</td>
<td>From 545 to 564</td>
<td>137 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GGGCACTTGAAGAGAAAGCA-3’</td>
<td>From 662 to 681</td>
<td>167 bp</td>
</tr>
<tr>
<td>MCT2</td>
<td>Forward 5’-CTCTTGTAAAGAGGATGGAGC-3’</td>
<td>From 1069 to 1090</td>
<td>352 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TAACCCCTGTCCTTCTTCCACT-3’</td>
<td>From 1399 to 1420</td>
<td>208 bp</td>
</tr>
<tr>
<td>MCT3</td>
<td>Forward 5’-GGAGAGGGCTGCTGGCCTCT-3’</td>
<td>From 1076 to 1094</td>
<td>231 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GGAGAGGGCTGCTGGCCTCT-3’</td>
<td>From 1261 to 1283</td>
<td>208 bp</td>
</tr>
<tr>
<td>MCT4</td>
<td>Forward 5’-CTCTTGGCTGCTGCTGCTGCTG-3’</td>
<td>From 2503 to 280</td>
<td>321 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GGAGAGGGCTGCTGGCCTCT-3’</td>
<td>From 558 to 579</td>
<td>321 bp</td>
</tr>
</tbody>
</table>

bp, base pair; Prod., product.
saponin. Then secondary antibody, Alexa 488 anti-rabbit for MCT1, MCT3, and MCT4 and Alexa 488 anti-chicken for MCT2 as well as Alexa 594 anti-rat were applied for 1 hour at room temperature. Slides were washed, counterstained with DAPI and mounted with anti-fade medium (Prolong; Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Fluorescence was observed with a standard epifluorescence microscope equipped with a cooled CCD camera.

Measurement of Intracellular pH

BCEC were cultured onto 25-mm glass coverslips and loaded with the pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) by incubation in BF Ringer solution containing 1 to 5 μM BCECF-AM at room temperature for 30 minutes. Dye-loaded cells were then kept in BF Ringer solution for at least 30 minutes before use. Fluorescence was excited alternately at 495 ± 10 and 440 ± 10 nm. Fluorescence emission (520–550 nm) ratios (F(495)/F(440)) obtained at 1 Hz were calibrated against pH, by the high-K⁺-ergicin technique.17 Coverslips were placed in a single-sided perfusion chamber.19 The assembled chambers were placed on a water-jacketed (37 °C) brass collar held on the stage of an inverted microscope (Diaphot 200; Nikon, Tokyo, Japan) and viewed with a long working distance (2 mm) water-immersion objective (×40; Nikon). The chamber was connected by tubing (Phar-Med; Saint-Gobain Performance Plastics, Paris, France) to hanging syringes that contained Ringer solution in a polymethyl methacrylate (Plexiglas; Altuglas, Philadelphia, PA) warming box (37 °C). The flow of the perfusate (approximately 0.5 mL per minute) was achieved by gravity. An eight-way valve was used to select the desired perfusate for the chamber.

Ringer Solutions and Chemicals

The composition of the standard BF Ringer used throughout this study was (in mM) 150 Na⁺, 4 K⁺, 0.6 Mg²⁺, 1.4 Ca²⁺, 118 Cl⁻, 1 HPO₄²⁻, 10 HEPES, 28.5 glucose, and 5 glucose. Bicarbonate-rich (BR) Ringer solution was prepared by equimolar substitution of sodium gluconate with NaHCO₃. Ringer solutions were equilibrated with air (BF) or 5% CO₂ (BR) and pH was adjusted to 7.50 with NaOH at 37 °C. L-lactate solutions were prepared on the morning of the experiment. Osmolarities of all solutions were adjusted to 295 to 300 mOsm by adding sucrose. Sodium L(+)-lactate, niflumic acid, 5-(Vethyl-Vispropyl)amiloride (EIPA), disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) and acetazolamide (ACTZ) were obtained from Sigma Aldrich (St. Louis, MO). BCECF-AM was purchased from Molecular Probes.

NBCe1 Knockdown

NBCe1 (SLC44A4) siRNA for bovine was purchased from Santa Cruz Technology (catalog # sc-270147). The siRNA contained the following three sequences: GGAAUAGGUGACCUAUCUtt, GUACAUCGGUG-GAGGAAUtt, and CAAGAAAGGAGGCUGAAUtt. Duplex-tandem dependent experiments were performed to determine optimal siRNA concentration and time after transfection that gives the most effective knockdown. After 4 days of transfection, 50 nm of NBCe1 siRNA was found to be most effective, producing greater than 90% knockdown. Transfection was performed in accordance with manufacturer’s protocol (Lipofectamine RNAiMAX; Invitrogen, Rockville, MD). For endogenous lactate efflux experiments with NBCe1 knockdown, BCEC were cultured in 12 mm Transwell inserts with 10% BCS and 1% antibiotic and/or antimycotic and placed in a 12-well culture plate. When the cells reached 50% to 60% confluency, they were transfected with 50 nm of siNBCe1 using 1 mL medium (Opti-MEM; Gibco, Invitrogen) and RNAiMAX lipofectamine for 4 hours. The siRNA medium was removed after 4 hours and replaced with DMEM with 10% BCS and 1% antibiotic and/or antimycotic. A nontargeting siRNA (Dharmacon, Thermo Fisher Scientific, Chicago, IL) was used as negative control. The DMEM was changed every other day. The experiment was performed 4 days posttransfection when the cells were confluent and NBCe1 knockdown was maximal, consistent with our previous study.20

Measurement of Endogenous Lactate Efflux

Endogenous lactate efflux is the measurement of the rate of lactate produced by the cells that has diffused across the cell membranes. The experiments are performed using tissue culture inserts so that the apical and BL efflux can be determined. The experiments were initially performed with confluent cultures on 10 mm Anopore tissue culture inserts, but later switched to 12 mm Transwell due to temporary unavailability of Anopores. Endogenous lactate efflux in BF and BR solution and in the presence of ACTZ was performed with Anopore inserts while all other endogenous lactate efflux experiments were performed with Transwell inserts. All experiments were performed in a plexiglass incubator warmed to 37 °C and gassed with 5% CO₂ when BR ringer was used. Anopore inserts were placed in a 12-well plate and bathed in 350 μL of DMEM in the inner compartment (apical) and 500 μL of DMEM in the outer compartment (basal). DMEM (500 μL) was used in the inner compartment and 1 mL in the outer for Transwell inserts. To measure the difference in endogenous lactate efflux under BF and BR conditions, the cells were first equilibrated in BF ringer solution for 20 minutes at 37 °C. After 20 minutes, the Ringer solution was removed and 350 μL of fresh Ringer solution were placed in the apical and basal compartment, respectively. Samples (5 μL) were taken from both compartments at 3, 5, and 10 minutes. The solution was then removed and replaced with BR Ringer and equilibrated in the incubator for 15 minutes. After 15 minutes, the solution was removed and replaced with fresh BR Ringer. Again, 5 μL samples were taken from both compartments at 3, 5, and 10 minutes. To test the effect of ACTZ on endogenous lactate efflux, 100 μM ACTZ in BR solution was added in both compartments and 5 μL samples were taken from the apical compartment at 3, 5, and 10 minutes. Samples taken with BR Ringer solution before the addition of ACTZ were used as control. Experiments performed with 250 μM DIDS and 1 μM EIPA were conducted using Transwell inserts. Samples (10 μL) were taken at 5, 10, and 20 minutes from cells in BR solution to serve as control. Then the cells were allowed to equilibrate with the inhibitors for 10 minutes. The solution was then removed and fresh BR ringer with inhibitors was added to both compartments and samples collected over the 20-minute period. Lactate content of collected samples was determined using a fluorometric lactate assay (Biovision, Mountain View, CA). The experiments described above were done sequentially with the same cells serving as controls first and then subjected to either bicine or experimental inhibitors. In the last experiment, endogenous lactate efflux was measured from cells treated with NBCe1 siRNA or negative control siRNA bathed in BR Ringer solution. Again, 10 μL samples were taken at 5, 10, and 20 minutes. Measurements from NBCe1 siRNA treated cells and control cells were conducted in parallel.

Statistical Analysis

Data analysis is presented as mean values ± SE. Paired t-test was used for statistical analysis and P < 0.05 was considered significant.

Results

Figure 1A shows RT-PCR results for MCT1–4 for the bovine RPE and corneal endothelium. As expected, a single band was detected for all four MCTs in the RPE sample. RT-PCR produced the expected band size for MCT1, 137 bp; MCT2, 352 bp; and MCT4, 321 bp. MCT3, however, was not detected in the endothelium. DNA sequencing of the PCR bands confirmed the presence of MCT1, -2, and -4 in bovine corneal endothelium. Figure 1B shows a Western blot analysis for MCT1–4 proteins in cultured BCEC. A bandsize of approximately 50 kDa was present with anti-MCT1, -2, and -4 antibodies. Anti-MCT3 antibody did not produce a positive band in BCEC, which is
consistent with the PCR results. Figure 2 shows immunofluorescence micrographs for MCT1, -2, and -4 using BCEC. MCT1 staining is clearly lateral, whereas MCT2 shows both lateral and apical staining and MCT4 is predominately apical. These results indicate that BCEC express MCT1, -2, and -4 and that they are distributed to both apical and BL membranes.

Next, we examined the effect of lactate on \( \text{pHi} \) in BCEC. BCEC were cultured onto 25-mm glass coverslips, loaded with BCECF and mounted into a perfusion chamber. The cells were perfused with lactate in BF Ringer for 20 seconds. Figure 3A shows that brief perfusion with 20 mM lactate produced rapid acidification that quickly returned to baseline \( \text{pHi} \) after lactate removal. Figure 3A also illustrates that when the cells were incubated with 100 \( \mu \text{M} \) niflumic acid, an MCT inhibitor,\(^5\) the average rate of \( \text{pHi} \) change and extent of acidification were reduced. Figure 3B shows that the rate was reduced by 65\% \((P < 0.001)\) and this was partially (87\%) reversible. Figure 3C shows that the amount of LIA was decreased by 0.26 pH unit \((P < 0.001; n = 7)\), in the presence of 100 \( \mu \text{M} \) niflumic acid. These results indicate that LIA was facilitated, consistent with previous reports using rabbit corneal endothelium.\(^4\)

In the absence of \( \text{HCO}_3^- \), buffering capacity is low; however, the influx of \( \text{H}^+ \) can activate \( \text{pHi} \) regulatory transporters, which have the potential to facilitate lactate/\( \text{H}^+ \) flux. To test if the lactate-induced \( \text{H}^+ \) influx activated \( \text{Na}^+ / \text{H}^+ \) exchange, cells were exposed to lactate for a longer duration. Figure 4A shows that lactate removal after a 2-minute exposure alkalinized the cells to a point above the baseline \( \text{pHi} \) (overshoot). To test our hypothesis that the overshoot after LIA is due to activation of the BL NHE1, we exposed the cells to lactate for 2 minutes in control conditions followed by lactate exposure in the presence of 1 \( \mu \text{M} \) EIPA, a NHE exchange inhibitor.\(^22\) Figure 4A shows that introduction of EIPA significantly reduced the overshoot seen after LIA; however, Figures 4B and 4C show that the acidification rate and depth of LIA were reduced, but these differences were not statistically significant \((P = 0.15 \text{ and } P = 0.17, \text{ respectively}; n = 5)\).

Next, we tested the hypothesis that increased cellular buffering capacity due to the presence of \( \text{HCO}_3^- \) and CA activity enhances lactate/\( \text{H}^+ \) flux in corneal endothelium. To do so, we measured LIA under BF and BR conditions and in the presence and absence of ACTZ, a CA inhibitor. Figure 5A shows that in the presence of \( \text{HCO}_3^- \), there is less LIA relative to that in BF ringer (Fig. 5B). Treating the cells with 100 \( \mu \text{M} \) ACTZ in the presence of \( \text{HCO}_3^- \) reduced the rate of acidification by 35\% but had no effect in BF. To estimate lactate-dependent proton flux under these conditions \( J_{\text{H}^+} \) (mM/sec) = \( \beta_T \)
dpH/dt, the total cellular buffering capacity, \( \beta_T \), must be known for each condition. \( \beta_T \) is the sum of intrinsic buffering, \( \beta_i \), and bicarbonate buffering (\( 2.303 \times [\text{HCO}_3^-] \)).\(^{23}\) Endothelial intrinsic buffering (\( \beta_i = 10 \text{ mM/pH} \)) was previously determined in bovine corneal endothelium.\(^{17}\) We used the initial rate of pH change taken over the first 20 seconds for dpH/dt. Table 2 shows that although the rate of LIA in BR was about half that in BF, because of the increased buffering capacity in BR, lactate-dependent proton flux was three times greater. Moreover, bicarbonate-dependent intracellular buffering was reduced by 41% in the presence of ACTZ. This relative reduction in buffering in the presence of ACTZ was experimentally determined by measuring the change in pH during a pulse of 5mM NH\(_4\)Cl in the absence and presence of 100 \( \mu \text{M} \) ACTZ while being perfused in BR Ringer (data not shown). Table 2 also shows that ACTZ had no effect on lactate-dependent proton flux in the absence of HCO\(_3^-\); however, in the presence of HCO\(_3^-\) proton flux decreased by 56%. These data indicate that lactate/H\(^+\) flux is enhanced by increased buffering capacity as supplied by HCO\(_3^-\) and CA activity.

To further examine the role of buffering as supplied by HCO\(_3^-\) and membrane transporters, we made direct measurements of endogenous apical and BL lactate efflux under BF and BR conditions using cultured BCEC grown on tissue culture inserts. BF or BR Ringer solution was placed in the apical (350 \( \mu \text{L} \)) and BL (500 \( \mu \text{L} \)) compartments and apical or basolateral samples were collected at various time periods (3, 5, and 10 minutes). Figure 6A shows that in either condition the endogenous efflux rate was approximately 50% greater into the apical side relative to BL. Combining all endogenous lactate flux experiments under control conditions, the lactate efflux into the apical compartment was 1.8-fold more than the BL compartment (\( P < 0.001; n = 52 \)). Comparing BR with BF, lactate efflux was 2.8-fold greater into the apical (\( P = 0.0001 \)) and 3.7-fold greater into the BL compartment (\( P = 0.008 \)) in the presence of HCO\(_3^-\) (\( n = 10 \)). Figure 6B shows that with the addition of 100 \( \mu \text{M} \) ACTZ in BR medium, endogenous lactate efflux into the apical compartment significantly decreased by 42% (\( P = 0.002; n = 9 \)).

Next we examined the potential contribution of buffering from membrane transport activity on lactate flux rates. First, we disrupted the transport of HCO\(_3^-\) by using DIDS, an anion transport inhibitor known to significantly block basolateral 1Na\(^+\):2HCO\(_3^-\) cotransport (NBCe1), the anion exchanger (AE2) and apical anion channels. Figure 7A shows that inhibiting HCO\(_3^-\) transport with DIDS decreased endogenous lactate efflux by 39% from 78 to 48 \( \mu \text{M per hour per cm}^2 \) on the apical surface and 44% from the basolateral surface.
43 ± 10 to 23 ± 10 μM per hour per cm² on the BL surface (n = 12). The reduction reached statistical significance on the apical surface (P = 0.02) but not on the basolateral surface (P = 0.28). In addition, we used siRNA specific to NBCe1 to reduce 1Na⁺.2HCO₃⁻ cotransport activity. Figure 7B shows that when NBCe1 was knocked down by siRNA, endogenous lactate efflux was 24 ± 2 μM per hour per cm² on the apical surface and 6 ± 1 μM per hour per cm² on the BL, which was 65% and 78%, respectively, lower than efflux in the control siRNA-treated cells (P = 0.001; n = 18).

Lastly, we examined endogenous lactate efflux in BR with cells exposed to EIPA. Figure 8 shows that EIPA reduced endogenous lactate efflux by 30% from 83 ± 8 to 58 ± 4 μM per hour per cm² (P = 0.001) on the apical surface and 54% from 18 ± 6 to 8 ± 2 (P = 0.10) on the BL surface (n = 12).

**DISCUSSION**

Lactate-dependent proton flux was previously demonstrated in rabbit corneal endothelium; however, the transport mechanism was unknown. Here we show that MCT1, MCT2, and MCT4 are expressed in the bovine corneal endothelium consistent with niflumic acid-sensitive lactate-induced cell acidification. Although niflumic acid is not a specific inhibitor of MCTs, we expect it to slow lactate-dependent acidification. In addition, we have shown that lactate-dependent cellular acidosis induces activation of Na⁺/H⁺ exchange and have demonstrated that the buffering contributed by HCO₃⁻, HCO₃⁻ transporters, NHE, and CA activity enhance lactate efflux from the corneal endothelium.

The distribution of a specific MCT isoform in a tissue is dependent on its physiological and metabolic needs. Each MCT isoform differs in its sensitivity to the various monocarboxylates and has different kinetic properties. MCT2 has a higher affinity for L-lactate (Kₘ = 0.7 mM) than MCT1 (Kₘ = 3 to 5 mM) and MCT4 (Kₘ = 28 mM). In our study, we found MCT1 and 2 localized on the basolateral surface and MCT4 and possibly MCT2 on the apical surface of the bovine corneal endothelium. The distribution of MCT1 and 2 on the BL surface potentially provides high affinity uptake of lactic acid. The major source of lactate on the basolateral surface is from the glycolytic anterior corneal epithelium and stromal keratocytes. Rapid uptake of lactic acid by the corneal endothelium provides efficient removal of lactate from the cornea allowing continued glycolysis and prevention of lactic acidosis. The presence of MCT(s) on the apical surface and the efflux of endogenous lactate into the apical compartment (Fig. 6) suggest that lactic acid taken up on the BL surface is expelled into the anterior chamber and this is consistent with whole cornea studies that showed ample lactate flux from cornea to anterior chamber. Further studies are needed to determine the contributions to this flux from the individual MCT isoforms.

Kinetic analysis of MCT transport has demonstrated a mechanism that involves proton binding first and that lactate may bind only to the transporter in the proton-bound state. At
The exact mechanism underlying the facilitation of lactate transport in the presence of CA is controversial. CA speeds the interconversion between CO$_2$ with HCO$_3^-$ and H$^+$. CAs are often positioned adjacent to HCO$_3^-$ transporters in a transport metabolon that would speed the local removal or availability of H$^+$ and HCO$_3^-$ \cite{29,33}. An alternative view is that CA acts non-catalytically to buffer H$^+$ \cite{10,15}. In the absence of CA, H$^+$ entering the cell via MCT1 or MCT4 accumulate in a microdomain at the inner face of the cell membrane dissipating the H$^+$ gradient and slowing lactate transport. In their Xenopus model, Becker et al. \cite{10,14,15} found that CA expression enhanced lactate/H$^+$ flux, but they saw a lack of effect of carbonic anhydrase inhibition suggesting that CA maintains the gradient by removing the H$^+$ at the pore of the MCT in a noncatalytic manner. Our data (Table 2) does not support this view. We found significant inhibition of LIA and reduced lactate efflux in the presence of ACTZ indicating the CA catalytic activity facilitated H$^+$ buffering.

In the endogenous lactate efflux experiments (Fig. 6), lactate produced by the CE cells can leave at either apical or basolateral membranes. However, efflux at the apical surface was favored (Fig. 6A). On the extracellular apical surface, the presence of HCO$_3^-$, anion channel-dependent HCO$_3^-$ efflux \cite{34} and CAIV activity \cite{18} can buffer the protons transported out of the cell thereby facilitating lactate/H$^+$ efflux. Conversely, at the BL surface, 1Na$^+$/2HCO$_3^-$ cotransport influx and lactate/H$^+$ efflux are going in opposite directions so the effective buffering is reduced. If MCT and NBCe1 were transporting in the same direction, this interpretation would predict greater lactate/H$^+$ uptake relative to efflux at the basolateral membrane. Further studies using separate basolateral perfusion and transendothelial lactate fluxes will be needed to test this notion.

Increasing the rate of epithelial lactate production (e.g., by contact lens wear-induced hypoxia), which in turn increases stromal [lactate] causes corneal edema due to the increased osmotic load within the cornea. \cite{2} Therefore, interfering with lactate removal will slow resolution of corneal edema. By extension, interfering with lactate removal in the normoxic cornea should cause corneal edema. Our results indicate that early studies showing the sensitivity of endothelial function to inhibitors of carbonic anhydrase \cite{35} Na$^+$/H$^+$ exchange, \cite{36} anion transport, and [HCO$_3^-$] need to be re-examined as to their direct effects on facilitating lactate removal because this could have substantial effects on corneal hydration control.

**FIGURE 7.** The role of bicarbonate transporters on lactate flux. (A) Inhibiting bicarbonate transport with 250 $\mu$M DIDS decreased endogenous lactate efflux by 39% on the apical surface ($P < 0.05$) and 44% on the basolateral surface ($n = 12$). (B) When NBCe1 was knocked down by siRNA, endogenous lactate efflux was 65% lower than efflux in the control siRNA-treated cells into the apical compartment and 78% lower into the basolateral compartment ($P < 0.05$; $n = 18$).

**FIGURE 8.** The role of NHE1 on lactate flux. Inhibiting NHE1 activity with 1 $\mu$M EIPA reduced endogenous lactate efflux by 30% on the apical surface ($P < 0.05$) and 54% on the basolateral surface ($n = 12$).
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References