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

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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 HCO₃⁻, 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 pH_i were measured in BCEC in HCO₃⁻-free or HCO₃⁻-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_i 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_i change (dpH_i/dt) and lactate-induced cell acidification. EIPA inhibited alkalinization after lactate removal. Lactate-dependent proton flux was significantly greater in the presence of HCO₃⁻ but was reduced by ACTZ. Efflux of endogenously produced lactate was significantly faster in the presence of HCO₃⁻, 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 HCO₃⁻, HCO₃⁻ transport, NHE and CA activity. (*Invest Ophthalmol Vis Sci.* 2011;52:8086–8093) DOI:10.1167/iov.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,¹ which results in a substantial [lactate] gradient between the cornea and aqueous humor.² Accumulation of lactate, for example induced by hypoxia, creates an osmotic load that leads to corneal edema.² Because the outer layers of corneal epithelium are impermeable to lactate,² 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.³ 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.⁴ Using rabbit corneas, Giasson and Bonanno⁴ 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.⁵ 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.^{6,7} MCT1 is the most widely distributed isoform 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.⁸ Lastly, MCT4 can be found in glycolytic tissues such as white skeletal muscle fibers, astrocytes, white blood cells, and chondrocytes. Chidlow et al.⁹ 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.^{10–16} Becker et al.¹³ reported that lactate transport activity by MCT1 is increased two-fold when coexpressed with the sodium bicarbonate cotransporter (NBC) in

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Supported by the National Institutes of Health Grants RO1EY08834 (JAB), K12EY015504, KO8EY019537 (TTN), P30EY019008, and an American Optometric Foundation Ezell Fellowship (AOF).

Submitted for publication June 20, 2011; revised July 26, 2011; accepted August 7, 2011.

Disclosure: T.T. Nguyen, None; J.A. Bonanno, None

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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 pH_i 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, *SLC9*) exists in the corneal endothelium and is active when pH_i 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.¹⁷ Primary culture was established in T-25 flasks with 5 mL Dulbecco modified Eagle medium (DMEM), 10% bovine calf serum (BCS) and 1% antibiotic/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 two 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 (AmpliTaQ 360 DNA Polymerase Protocol; Applied Biosystems, Rockville, MD) using primers specific to the MCT1-4 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 report⁹ 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 (1:5000 dilution). Bound antibody was detected (Supersignal West Pico Chemiluminescent substrate; Pierce).

Immunofluorescence

Immunofluorescent staining was performed on BCEC cultured on 25 mm coverslips as previously described.¹⁸ 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 0.01%

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

Gene	Nucleotide Sequences	Positions (at cDNA)	PCR Prod. Size
<i>MCT1</i>	Forward 5'-GGAGTCATTGGAGGTCTTGG-3'	From 545 to 564	137 bp
	Reverse 5'-GCCAGGGTAGAGAGGAACAC-3'	From 662 to 681	
<i>MCT2</i>	Forward 5'-CTCTTTGAAACGCTCATGGACC-3'	From 1069 to 1090	352 bp
	Reverse 5'-TAACCTCTGCTCCTTCCACTC-3'	From 1399 to 1420	
<i>MCT3</i>	Forward 5'-TGCACTTCGAGGTGCTCAT-3'	From 1076 to 1094	208 bp
	Reverse 5'-TTGGTGGCCACAGCCATGAAGAT-3'	From 1261 to 1283	
<i>MCT4</i>	Forward 5'-CATCCTTTGGGGCTGTTTCGTC-3'	From 259 to 280	321 bp
	Reverse 5'-AAGTTGAGCGCCAAACCCAGC-3'	From 558 to 579	
β-actin	Forward 5'-CGTGAGAAGATGACCCAGATCATG-3'	From 385 to 408	300 bp
	Reverse 5'-CTTCTCCTTGATGTACGGACGAT-3'	From 661 to 684	

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 acetoxymethyl ester (BCECF-AM) by incubation in BF Ringer solution containing 1 to 5 μ M 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_i by the high-K⁺-nigericin 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 ($\times 40$; 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 HPO₄^{2−}, 10 HEPES, 28.5 gluconate[−], 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-(N-ethyl-N-isopropyl)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 (*SLC4A4*) siRNA for bovine was purchased from Santa Cruz Technology (catalog # sc-270147). The siRNA contained the following three sequences: GGAAUAGGUGACCUAUACUtt, GUACUCAGGUGAGAGAAUtt, and CAAGAAAGGAGCUGGAUUAUtt. Dose- and time-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 μ L of DMEM in the inner compartment (apical) and 500 μ L of DMEM in the outer compartment (basal). DMEM (500 μ L) 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 μ L and 500 μ L of fresh Ringer solution were placed in the apical and basal compartment, respectively. Samples (5 μ L) 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 μ L samples were taken from both compartments at 3, 5, and 10 minutes. To test the effect of ACTZ on endogenous lactate efflux, 100 μ M ACTZ in BR solution was added in both compartments and 5 μ L 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 μ M DIDS and 1 μ M EIPA were conducted using Transwell inserts. Samples (10 μ L) 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 bicarbonate 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 μ L 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 $\pm$ 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

FIGURE 1. Expression of MCTs in bovine corneal endothelium. **(A)** Detection of MCT1–4 mRNA using RT-PCR. Negative control (–) represents cDNA samples not subjected to reverse transcriptase. RPE serves as a positive control. For each reaction, amplification yielded a single band whose size was equivalent to that predicted from the relevant sequence. **(B)** Detection of MCT-4 proteins in cultured BCEC by Western blot analysis. Whole cell lysates from bovine corneal endothelium were resolved using 12% SDS-PAGE gel. Total protein (30 μ g) was loaded per lane. MCT1, 2, and 4, but not MCT3, were immunodetected at approximately 50 kDa.

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 pH_i 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 pH_i after lactate removal. Figure 3A also illustrates that when the cells were incubated with 100 μ M niflumic acid, an MCT inhibitor,^{5,21} the average rate of pH_i 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 (from -0.46 ± 0.06 to -0.20 ± 0.03 pH unit; $P < 0.001$; $n = 7$), in the presence of 100 μ M niflumic acid. These results indicate that LIA was facilitated, consistent with previous reports using rabbit corneal endothelium.⁴

In the absence of HCO_3^- , buffering capacity is low; however, the influx of H^+ can activate pH_i regulatory transporters, which have the potential to facilitate lactate/ H^+ flux. To test if the lactate-induced H^+ influx activated Na^+/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 pH_i (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 μ 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 ($P = 0.15$ and $P = 0.17$, respectively; $n = 5$).

Next, we tested the hypothesis that increased cellular buffering capacity due to the presence of HCO_3^- and CA activity enhances lactate- 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 HCO_3^- , there is less LIA relative to that in BF Ringer (Fig. 5B). Treating the cells with 100 μ M ACTZ in the presence of HCO_3^- reduced the rate of acidification by 35% but had no effect in BF. To estimate lactate-dependent proton flux under these conditions $[J_{H^+} \text{ (mM/sec)} = \beta_T$

FIGURE 2. Immunohistochemical localization of MCT1, -2, and -4. Representative immunofluorescent images for MCT1, -2, and -4 in cultured BCEC; **(A)** control, **(B)** MCT1, **(C)** MCT2, and **(D)** MCT4. Control samples were not subjected to the respective MCT primary antibodies. Overlay images: blue, nuclei; red, ZO-1; green, MCT. MCT1 staining is clearly lateral, whereas MCT2 shows both lateral and apical staining and MCT4 is predominately apical.

FIGURE 3. Effect of niflumic acid on lactate-induced acidification. (A) Representative trace showing effect of 100 μ M niflumic acid on LIA. Arrowheads indicate where lactate pulses were given. (B) Bar graph quantifies the mean acidification rates and SE ($n = 7$ coverslips). Rates were determined over the first 20 seconds. The mean rate was reduced by 65% with niflumic acid and this was partially (87%) reversible. (C) Bar graph shows the mean change in pH_i and SE. Lactate-induced acidification was decreased by 0.26 pH unit in the presence of niflumic acid. * Significantly lower than control ($P < 0.05$).

dpH_i/dt], the total cellular buffering capacity, β_T , must be known for each condition. β_T is the sum of intrinsic buffering, β_i , and bicarbonate buffering ($=2.303 \cdot [HCO_3^-]_i$).²³ Endothelial intrinsic buffering ($\beta_i = 10$ mM/pH) was previously determined in bovine corneal endothelium.¹⁷ We used the initial rate of pH_i change taken over the first 20 seconds for dpH_i/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_i during a pulse of 5mM NH_4Cl in the absence and presence of 100 μ 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 μ L) and BL (500 μ 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 μ 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 ± 13 to 48 ± 10 μ M per hour per cm^2 on the apical surface and 44% from

FIGURE 4. The role of NHE1 on alkalinization over baseline (overshoot) after LIA. (A) Lactate pulses were given for approximately 2 minutes. The arrows indicate the overshoot. The break in the curve represents a time interval in which the cells were incubated in 1 μ M EIPA. (B) Mean rates and SE ($n = 5$). Rates were determined over the first 20 seconds (C) Average change in pH_i and SE. EIPA significantly reduced the overshoot seen after LIA; however, both the rates and amount of pH_i change were not statistically ($P > 0.05$) affected by EIPA.

FIGURE 5. The effect of increased cellular buffering capacity by bicarbonate and carbonic anhydrase activity on lactate-dependent proton flux. Representative traces of the change in pH_i induced by 20 mM lactate in (A) BR and (B) BF medium, and in the absence and presence of 100 μM ACTZ. Arrows indicate where lactate pulses were given.

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 63% 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 demon-

FIGURE 6. The role of bicarbonate and carbonic anhydrase on lactate flux in BF and BR solution and in the presence and absence of ACTZ. (A) Endogenous lactate efflux in BF and BR solution for the apical and basolateral compartments. * Statistically greater (*P* < 0.05; *n* = 11) lactate efflux in BR compared with BF. (B) Apical lactate efflux in the presence of 100 μM ACTZ. * Statistically decreased (*P* < 0.05; *n* = 9) in endogenous lactate efflux in the presence of ACTZ.

strated 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 (*K_m* = 0.7 mM) than MCT1 (*K_m* = 3 to 5 mM) and MCT4 (*K_m* = 28 mM).⁷ 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 highly glycolytic anterior corneal epithelium and stromal keratocytes.¹ 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.² 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.²⁴ At

TABLE 2. Lactate-Dependent H⁺ Flux

	β _i	β _{HCO3}	β _T	δpHi/dt	j _{H+} (mM/s)
BF	10	0	10	0.03	0.3
BR	10	46	56	0.017	0.95
BF with ACTZ	10	0	10	0.03	0.0.3
BR with ACTZ	10	27	37	0.011	0.41

FIGURE 7. The role of bicarbonate transporters on lactate flux. (A) Inhibiting bicarbonate transport with 250 μM 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 63% lower than efflux in the control siRNA-treated cells into the apical compartment and 78% lower into the basolateral compartment ($P < 0.05$; $n = 18$).

10^{-7} M, H^+ binding is the rate-limiting step. Increased concentrations of mobile buffers can significantly increase the availability and apparent mobility of H^+ .^{25,26} Moreover, because buffering is significantly faster in the presence of HCO_3^- , coupling lactate/ H^+ cotransport to HCO_3^- /CA buffering would be an efficient transport system. Consistent with this notion, transport activity of MCTs in a number of cell types is enhanced by $1\text{Na}^+ : 2\text{HCO}_3^-$ cotransporter (NBC) and CA activity.^{11-14,27} In our study, we asked if buffering from HCO_3^- and CA activity, together with membrane transporters could facilitate lactate dependent H^+ flux and lactate flux directly. We demonstrated an increased rate of LIA in the presence of HCO_3^- that was facilitated by CA activity (Fig. 5) and increased endogenous lactate efflux facilitated by HCO_3^- and CA activity (Fig. 6). The effectiveness of intracellular bicarbonate buffering is also dependent on Na^+/H^+ exchange and $1\text{Na}^+ : 2\text{HCO}_3^-$ cotransport activity. Inhibiting these transporters also slowed lactate efflux. Intracellular pH is not affected by Na^+/H^+ exchange inhibition in BCEC⁴ and only slightly decreased (approximately 0.2 pH unit) by NBCe1 inhibition,^{17,20} indicating that the driving force for lactate/ H^+ efflux would be unchanged or slightly increased, respectively. Yet our results showed a decrease in lactate/ H^+ efflux in both conditions. These results are consistent with lactate/ H^+ efflux being predominately affected by buffering capacity (i.e., the availability and mobility of H^+) and not by small changes in the bulk H^+ driving force. Consistent with this conclusion, a greater effect was seen with inhibition of NBCe1 activity because this transporter has a greater effect on pH_i regulation and buffer capacity.²⁸

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^- .²⁹⁻³³ An alternative view is that CA acts non-catalytically to buffer H^+ .^{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.^{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^- efflux³⁴ and CAIV activity¹⁸ can buffer the protons transported out of the cell thereby facilitating lactate/ H^+ efflux. Conversely, at the BL surface, $1\text{Na}^+ : 2\text{HCO}_3^-$ 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.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,³⁵ Na^+/H^+ exchange,³⁶ anion transport, and $[\text{HCO}_3^-]$ ³⁷ 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 8. The role of NHE1 on lactate flux. Inhibiting NHE1 activity with 1 μM EIPA reduced endogenous lactate efflux by 30% on the apical surface ($P < 0.05$) and 54% on the basolateral surface ($n = 12$).

Acknowledgments

The authors thank Shimin Li for technical assistance with RT-PCR.

References

- Riley MV. Glucose and oxygen utilization by the rabbit cornea. *Exp Eye Res.* 1969;8:193–200.
- Klyce SD. Stromal lactate accumulation can account for corneal oedema osmotically following epithelial hypoxia in the rabbit. *J Physiol.* 1981;321:49–64.
- Bonanno JA. Lactate-proton cotransport in rabbit corneal epithelium. *Curr Eye Res.* 1990;9:707–712.
- Giasson C, Bonanno JA. Facilitated transport of lactate by rabbit corneal endothelium. *Exp Eye Res.* 1994;59:73–81.
- Poole RC, Sansom CE, Halestrap AP. Studies of the membrane topology of the rat erythrocyte H⁺/lactate cotransporter (MCT1). *Biochem J.* 1996;320:817–824.
- Price NT, Jackson VN, Halestrap AP. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. *Biochem J.* 1998;329:321–328.
- Halestrap AP, Meredith D. The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch.* 2004;447:619–628.
- Philp NJ, Yoon H, Lombardi L. Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. *Am J Physiol Cell Physiol.* 2001;280:C1319–C1326.
- Chidlow G, Wood JP, Graham M, Osborne NN. Expression of monocarboxylate transporters in rat ocular tissues. *Am J Physiol Cell Physiol.* 2005;288:C416–C428.
- Becker HM, Deitmer JW. Nonenzymatic proton handling by carbonic anhydrase II during H⁺-lactate cotransport via monocarboxylate transporter 1. *J Biol Chem.* 2008;283:21655–21667.
- Wetzel P, Hasse A, Papadopoulos S, Voipio J, Kaila K, Gros G. Extracellular carbonic anhydrase activity facilitates lactic acid transport in rat skeletal muscle fibres. *J Physiol.* 2001;531:743–756.
- Svichar N, Chesler M. Surface carbonic anhydrase activity on astrocytes and neurons facilitates lactate transport. *Glia.* 2003;41:415–419.
- Becker HM, Broer S, Deitmer JW. Facilitated lactate transport by MCT1 when coexpressed with the sodium bicarbonate cotransporter (NBC) in *Xenopus* oocytes. *Biophys J.* 2004;86:235–247.
- Becker HM, Hirnet D, Fecher-Trost C, Sultemeyer D, Deitmer JW. Transport activity of MCT1 expressed in *Xenopus* oocytes is increased by interaction with carbonic anhydrase. *J Biol Chem.* 2005;280:39882–39889.
- Becker HM, Klier M, Deitmer JW. Nonenzymatic augmentation of lactate transport via monocarboxylate transporter isoform 4 by carbonic anhydrase II. *J Membr Biol.* 2010;234:125–135.
- Becker HM, Deitmer JW. Voltage dependence of H⁺ buffering mediated by sodium bicarbonate cotransport expressed in *Xenopus* oocytes. *J Biol Chem.* 2004;279:28057–28062.
- Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. I. Na⁺/H⁺ exchange in the absence and presence of HCO₃⁻. *Invest Ophthalmol Vis Sci.* 1992;33:3058–3067.
- Sun XC, Li J, Cui M, Bonanno JA. Role of carbonic anhydrase IV in corneal endothelial HCO₃⁻ transport. *Invest Ophthalmol Vis Sci.* 2008;49:1048–1055.
- Bonanno JA, Yi G, Kang XJ, Srinivas SP. Reevaluation of Cl⁻/HCO₃⁻ exchange in cultured bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 1998;39:2713–2722.
- Li J, Sun XC, Bonanno JA. Role of NBC1 in apical and basolateral HCO₃⁻ permeabilities and transendothelial HCO₃⁻ fluxes in bovine corneal endothelium. *Am J Physiol Cell Physiol.* 2005;288:C739–C746.
- Jackson VN, Halestrap AP. The kinetics, substrate, and inhibitor specificity of the monocarboxylate (lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein. *J Biol Chem.* 1996;271:861–868.
- Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of Na⁺/H⁺ exchanger. *Eur J Med Chem.* 2003;38:547–554.
- Roos A, Boron WF. Intracellular pH. *Physiol Rev.* 1981;61:296–434.
- Vinnakota KC, Beard DA. Kinetic analysis and design of experiments to identify the catalytic mechanism of the monocarboxylate transporter isoforms 4 and 1. *Biophys J.* 2011;100:369–380.
- Swietach P, Zaniboni M, Stewart AK, Rossini A, Spitzer KW, Vaughan-Jones RD. Modelling intracellular H⁺ ion diffusion. *Prog Biophys Mol Biol.* 2003;83:69–100.
- Zaniboni M, Swietach P, Rossini A, Yamamoto T, Spitzer KW, Vaughan-Jones RD. Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig. *Am J Physiol Heart Circ Physiol.* 2003;285:H1236–H1246.
- Svichar N, Esquenazi S, Waheed A, Sly WS, Chesler M. Functional demonstration of surface carbonic anhydrase IV activity on rat astrocytes. *Glia.* 2006;53:241–247.
- Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. II. Na⁺:HCO₃⁻ cotransport and Cl⁻/HCO₃⁻ exchange. *Invest Ophthalmol Vis Sci.* 1992;33:3068–3079.
- Alvarez BV, Vilas GL, Casey JR. Metabolon disruption: a mechanism that regulates bicarbonate transport. *EMBO J.* 2005;24:2499–2511.
- McMurtree HL, Cleary HJ, Alvarez BV, et al. The bicarbonate transport metabolon. *J Enzyme Inhib Med Chem.* 2004;19:231–236.
- Sowah D, Casey JR. An intramolecular transport metabolon: fusion of carbonic anhydrase II to the COOH terminus of the Cl⁻/HCO₃⁻ Formula exchanger, AE1. *Am J Physiol Cell Physiol.* 2011;301:C336–C346.
- Sterling D, Alvarez BV, Casey JR. The extracellular component of a transport metabolon. Extracellular loop 4 of the human AE1 Cl⁻/HCO₃⁻ exchanger binds carbonic anhydrase IV. *J Biol Chem.* 2002;277:25239–25246.
- Sterling D, Reithmeier RA, Casey JR. A transport metabolon. Functional interaction of carbonic anhydrase II and chloride/bicarbonate exchangers. *J Biol Chem.* 2001;276:47886–47894.
- Li J, Allen KT, Sun XC, Cui M, Bonanno JA. Dependence of cAMP mediated increases in Cl⁻ and HCO₃⁻ permeability on CFTR in bovine corneal endothelial cells. *Exp Eye Res.* 2008;86:684–690.
- Hodson S, Miller F. The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. *J Physiol.* 1976;263:563–577.
- Liebovitch LS, Fischbarg J. Effects of inhibitors of passive Na⁺ and HCO₃⁻ fluxes on electrical potential and fluid transport across rabbit corneal endothelium. *Curr Eye Res.* 1982;2:183–186.
- Hodson S. The regulation of corneal hydration by a salt pump requiring the presence of sodium and bicarbonate ions. *J Physiol.* 1974;236:271–302.