



Published in final edited form as:

Exp Eye Res. 2020 October ; 199: 108193. doi:10.1016/j.exer.2020.108193.

Bicarbonate Activates Glycolysis and Lactate Production in Corneal Endothelial Cells by Increased pH_i

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Abstract

Recent studies have shown that lactate coupled water flux is the underlying mechanism of the corneal endothelial pump, which is highly dependent on the presence of bicarbonate. In this study we test the hypothesis that the increased intracellular pH (pH_i) caused by bicarbonate stimulates glycolytic activity and the production of lactate by endothelial cells. Primary cultures of bovine corneal endothelial cells (BCEC) were incubated in bicarbonate-free (BF) ringer, a high [HEPES] ringer, and bicarbonate-rich (BR) ringer all at pH 7.5. Lactate production and glucose consumption were greatest in BR>HEPES >BF. Similarly, pH_i was greatest in BR>HEPES>BF. Increasing pH_i with NH_4Cl also increased lactate production in BF or BR, indicating that the increased lactate production in BR is not due to HCO_3^- itself. Glucose transport capacity, as measured by 2-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino-2-Deoxyglucose (2-NBDG) uptake was unaffected by the three incubation conditions. Using Laconic, a FRET sensor for lactate, we found that intracellular [lactate] increased immediately and transiently when cells were switched from BF to BR perfusion indicating increased lactate production with subsequent matching of efflux. Moreover, induction of acute lactate influx by perfusion pulses of 10 mM lactate increased intracellular [lactate] significantly faster in BF than in BR, consistent with higher lactate production and efflux in BR. In summary, our results indicate that glycolytic flux and lactate production increase in BR due to increased pH_i , consistent with the well-known pH sensitivity of phosphofructokinase, the rate limiting enzyme in glycolysis.

Keywords

Corneal endothelium; bicarbonate; intracellular pH; lactate production; glycolysis

The corneal endothelium is responsible for maintaining the transparency of the cornea by controlling stromal hydration. Steady-state hydration is achieved by the ion-linked fluid transport properties of the endothelium that offset stromal swelling pressure driven water influx. Primary active transport (Na^+K^+ ATPase), located on the lateral membrane of endothelial cells, provides Na^+ gradients that are required to drive basolateral $Na^+:2HCO_3^-$ cotransport and Na^+/H^+ exchange, the two major membrane transporters that control

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intracellular pH (pH_i) in corneal endothelium (Bonanno and Giasson, 1992a, c; Diecke et al., 2004; Li et al., 2005). Regulation of pH_i is strongly influenced by the robust buffering power of bicarbonate, which is supported by several carbonic anhydrases located on basolateral and apical membranes and within the cytosol (Cui et al., 2002; Fischbarg and Lim, 1974; Kuang et al., 1990; Terashima et al., 1996). These processes help drive lactate: H^+ cotransport uptake on the basolateral membrane and efflux apically to the anterior chamber. Inhibiting the Na^+ - K^+ ATPase (Anderson and Fischbarg, 1978; Li et al., 2016; Li et al., 2020; Riley et al., 1994), removing bicarbonate or inhibiting carbonic anhydrase activity (Fischbarg and Lim, 1974; Li et al., 2016; Li et al., 2020; Riley et al., 1995), inhibiting the Na^+ : 2HCO_3^- cotransporter (Bonanno and Giasson, 1992c; Nguyen and Bonanno, 2011) or Na^+ / H^+ exchanger (Liebovitch and Fischbarg, 1982; Nguyen and Bonanno, 2011), lowering extracellular buffering capacity (Li et al., 2016), shRNA knock down of monocarboxylate cotransport expression (MCTs) (Li et al., 2014), or pharmacologic inhibition of MCT cotransport (Li et al., 2016) all cause relative decreases in endothelial lactate flux with concomitant corneal edema. These findings led to the hypothesis that the corneal endothelial pump maintains hydration by linking water efflux to lactate efflux. The model (see Figure 1) (Li et al., 2016) predicts that endothelial dysfunction will reduce lactate efflux leading to increased corneal [lactate] and corneal edema, which was confirmed in rabbits (Li et al., 2016; Li et al., 2014) and in the *Slc4a11* knock out mouse model of CHED (Congenital Hereditary Endothelial Dystrophy) (Li et al., 2020).

Eighty-five percent of the glucose taken up by the cornea is metabolized to lactate (Riley, 1969), indicating that the cornea is highly glycolytic. The epithelium and stromal keratocytes are responsible for most of the lactate production, consistent with a relatively lower density of mitochondria compared with endothelial cells (Hogan et al., 1971). Lactate does not diffuse anteriorly (Klyce, 1981), so it must all diffuse posteriorly across the endothelium providing substrate for the pump mechanism. Seminal studies showed that pump function is unchanged when the epithelium is removed, proving that the endothelium is responsible for the pump, not the epithelium (Dikstein and Maurice, 1972; Riley et al., 1995). Epithelial removal significantly reduced corneal lactate production by 50% (Li et al., 2020). However, the linkage between lactate efflux and water efflux was unchanged, suggesting that the endothelium can form the basolateral to apical [lactate] gradients needed for osmotic water flux as long as some lactate is present.

Confluent monolayers of primary cultures of bovine corneal endothelial cells have been shown to transport water in the absence of epithelial cells or keratocytes (Kuang et al., 2004; Narula et al., 1992). The fluid transport rate was ~50-60% of that measured in intact corneas and was eliminated by inhibition of the Na^+ - K^+ ATPase or bicarbonate removal. These cultures also produce lactate where apical efflux is approximately twice basolateral efflux and the flux is dependent on bicarbonate, carbonic anhydrase activity, and the pH_i regulatory transporters (Nguyen and Bonanno, 2011, 2012). These studies suggest that the endothelium by itself retains some intrinsic water pumping capability that is dependent on bicarbonate and linked to lactate flux. Regulation of glycolysis and therefore production of lactate is primarily via phosphofructokinase (PFK). Classic studies have shown that PFK activity increases with increasing pH (Kitajima et al., 1983; Trivedi and Danforth, 1966). In the

current study, we test the hypothesis that lactate production by cultured bovine corneal endothelial cells is enhanced in bicarbonate due to the effects of pH_i on glycolytic activity.

METHODS

Cell culture

Fresh bovine eyes were obtained from a local slaughterhouse and corneal endothelial cells were harvested as previously described (Bonanno and Giasson, 1992a). Primary culture was established in T-25 flasks with 5 ml Dulbecco Modified Eagle Medium (DMEM), 10% bovine calf serum, and 1% antibiotic/antimycotic (penicillin 100 U/mL, and fungizone 0.25 $\mu\text{g}/\text{mL}$), gassed with 5% CO_2 -95% air at 37° C and medium changed every 2-3 days. All bovine corneal endothelial cells (BCEC) used in experiments are passage P1 – P3.

Ringer solutions and chemicals.

The composition of the standard bicarbonate-free Ringer (BF) used throughout this study was (in mM) 160 Na^+ , 4 K^+ , 0.6 Mg^{2+} , 1.4 Ca^{2+} , 102 Cl^- , 1 HPO_4^{2-} , 60 gluconate, and 5 glucose. HEPES Ringer was identical except that 60 mM Na-HEPES was substituted for sodium gluconate. Bicarbonate-rich ringer solution (BR) was prepared by equimolar substitution of sodium gluconate with 28.5 mM NaHCO_3^- . Ringer solutions were equilibrated with air (BF & HEPES) or 5% CO_2 (BR) and pH was adjusted to 7.50 with NaOH at 37°C. Lactate solutions were prepared on the morning of the experiment by equimolar substitution of Na^+ -gluconate by Na^+ -L(+) lactate. Osmolarities of all solutions were adjusted to 295-300 mOsmol by adding sucrose. Na^+ L(+) lactate, nigericin, and iodoacetate were obtained from Sigma Aldrich (St. Louis, MO). The pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was purchased from Molecular Probes (Thermo Fisher Scientific). 2-NBDG was obtained from (Sigma).

Lactate Production & Glucose Consumption

Primary cultured BCEC were subcultured to 12 well plates at 2×10^5 cells/well and grown to confluency within 3-5 days. Cells were washed with PBS and 0.5 ml of Ringer solutions (BF, HEPES or BR) was added. BF and HEPES plates were maintained at 37°C in air, and BR plates were maintained at 37°C and connected to 5% CO_2 . For measuring lactate production, a 50 μl sample was taken at 30, 60, 90, 120 and 150 minutes respectively, while replenishing with a 50 μl of Ringers at the same time. In order to measure glucose consumption, the glucose concentration was reduced to 0.1 mM in all ringer solutions, and samples were taken at 1, 2, 3, 4 and 5 hours respectively. Measurements of lactate and glucose were carried out by using assay kits from BioVision (Milpitas, CA).

Oxygen Consumption

Primary bovine corneal endothelial cells were seeded at 5×10^4 cells/well (250 μl volume) in 24-well plates (Agilent 100777-004) and grown to confluence in DMEM, 10% bovine calf serum, and 1% antibiotic/antimycotic (penicillin 100 U/mL, and fungizone 0.25 $\mu\text{g}/\text{mL}$), gassed with 5% CO_2 -95% air at 37° C. The day of the experiments, cells were washed and incubated in BF or HEPES 60 mM ringers for 2 hours at 37 ° C in a non- CO_2 incubator.

Mito Stress kit for Seahorse XFe24 (Agilent #103015-100) was run using reagents prepared in BF or HEPES 60 mM: Oligomycin 1.5 μ M, FCCP 0.5 μ M, Rotenone 0.5 μ M Antimycin A: 0.5 μ M. Data analysis was performed with Wave Software and Multi-File Report Generator. At the end of the assay, cells were trypsinized and counted in a Cellometer. Oxygen consumption rates were normalized to cell number.

Glucose Transport Capacity

Bovine primary corneal endothelial cells were incubated with BR, BF or HEPES ringers containing 10 μ M 2-NBDG for 30 mins, followed by dissociation with 0.25% Trypsin EDTA. Cells were washed with the respective buffers containing 5% FBS to neutralize trypsin and centrifuged at 4000g for 5 mins. Cell pellets were re-suspended in BF, BR or HEPES and filtered using CellTrics filters (Sysmex, Germany). A glucose transporter (Glut1) inhibitor, WZB117 (Tocris, Bristol, U.K) 20 μ M in BR solution, was used as a negative control for 2-NBDG uptake, and unstained cells were used to gate for autofluorescence. Flow cytometry analysis was conducted on MACSQuant VYB (Miltenyi Biotech, Germany). Immediately prior to analysis, 7-ADD reagent (Bio Legend, San Diego, CA) was added to all samples to stain dead cells, which were excluded. 10,000 cells were used for each acquisition, and treatment conditions were analyzed in triplicate. Data were analyzed using Flow-Jo software (FlowJo LLC., Ashland, OR).

Measurement of Intracellular pH

Bovine corneal endothelial cells were cultured onto 25-mm round glass coverslips and loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubation in BF Ringer solution containing 2 μ M BCECF-AM at room temperature for 30 min. Dye-loaded cells were then kept in BF Ringer solution for at least 30 minutes before use. Coverslips were placed in a single-sided perfusion chamber (Bonanno and Giasson, 1992a). 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 an oil immersion objective (40x; Nikon). Hanging syringes with Ringer solution were placed in a Plexiglas warming box (37°C) and connected to an eight-way Hamilton valve used to select the desired ringer. The flow of the perfusate (approximately 0.5 mL/min) was achieved by gravity. Connections to the valve and the valve to the chamber used Phar-Med tubing (Saint-Gobain Performance Plastics, Paris, France). BCECF 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 (Bonanno and Giasson, 1992b). Excitation light and data collection was controlled by PTI Felix software.

Intracellular Lactate

Relative changes in intracellular [Lactate] were measured using the lactate sensitive FRET probe Laconic (addgene.org, Cat# 44238) [27]. This probe was transiently transfected into primary cultured bovine corneal endothelial cells (BCEC) by using Lipofectamine 3000 (Life technologies, Cat# L3000-008). In brief, 5×10^5 BCEC were seeded on 25 mm diameter coverslips, placed in 6-well plates. 2 μ g of laconic plasmid was mixed with 10 μ l of transfection reagent, and the transfection complex was brought to a volume of 0.75 ml by

adding serum-free DMEM before being applied to the cells that have grown to ~60-70% confluence. After a 3-hour transfection the cells were grown in normal conditions for another 24 ~ 48 hour. The coverslip was mounted in the perfusion chamber, fluorescent cells were located and perfused with BF, HEPES, or BR. In some experiments 10 mM Na-lactate (substituted for Na-gluconate) was added. Fluorescence was excited at 440 nm and dual emission (490 and 520 nm) collected from transfected cells at 1 Hz. Excitation and data collection was controlled by PTI Felix software.

Statistical analysis

Summary data is presented as mean values \pm standard deviations. Student's *t*-test (paired or independent) was used to test for significance ($p < 0.05$). Bonferroni's multiple comparisons.

RESULTS

Lactate Production

Previous studies have shown that steady-state pH_i of corneal endothelial cells is higher in bicarbonate-rich ringer (BR) than in bicarbonate-free (BF) (Bonanno and Giasson, 1992a, c). To test the effect of pH_i on lactate production, primary cultures of bovine corneal endothelial cells (BCEC) were washed with PBS and incubated with BF, 60 mM HEPES buffered bicarbonate-free (HEPES), or BR ringer. Each experiment was done in triplicate and repeated with new cultures on three separate days. Figure 2A shows that lactate production was greatest in BR, then HEPES, and least in BF over 120 minutes. To measure glucose consumption rates under the same conditions, we had to lower the starting [glucose] in order to bring the [glucose] of samples within the assay range. Figure 2B shows that glucose consumption was greatest in BR, then HEPES, and least in BF, consistent with the differences in lactate production. These results suggest that glycolysis is accelerated in BCEC under conditions of higher pH_i . To confirm that this effect is via glycolysis and not from tricarboxylic acid cycle (TCA) intermediates feeding back to lactate production, the same experiments were repeated in the presence of 100 μM iodoacetate, a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor. Figure 2C shows that iodoacetate reduced lactate production to less than 10% under all three conditions.

Oxygen Consumption

The end product of glycolysis, pyruvate, can be converted to lactate or it can enter mitochondria and contribute to the TCA cycle and ultimately consume oxygen. Increased lactate production implies that the availability of pyruvate is also increased, some of which could enter mitochondria and increase oxygen consumption. To test this possibility, we measured oxygen consumption. Figure 2D shows that the basal oxygen consumption rate (OCR) is greater in HEPES than BF. (BR with CO_2 equilibration was not possible within the Seahorse machine). Moreover, ATP related oxygen consumption (Figure 2E) was also higher in HEPES, consistent with greater oxidative flux in endothelial cells with greater glycolytic activity.

Glucose Transport

We next asked if facilitated glucose uptake via Glut transporters is affected differentially by BR, HEPES, and BF incubation. We used 2-NBDG, a non-metabolizable fluorescent glucose analog (Zou et al., 2005), uptake assay to test this possibility. Figure 2F shows that there was no difference in the capacity to bring glucose into the cells under the three conditions.

Intracellular pH_i

Next, we measured pH_i under the three conditions to confirm that BR has higher pH_i than BF and to also test if HEPES incubation is intermediate. Figure 3A shows the calibration of fluorescence ratio (Ex495/440, Em 520-540) to pH_i. The average steady-state pH_i was 7.038 ± 0.091 in BF, 7.116 ± 0.118 in HEPES, and 7.226 ± 0.104 in BR. Figure 3B shows a representative trace of pH_i of BCEC perfused with BF, then switched to HEPES, then BR. The average of the paired differences between HEPES & BF ($+0.074 \pm 0.045$) and BR & BF ($+0.197 \pm 0.067$) were significantly different (n=5 pairs, p<0.05) confirming the rank order of pH_i as BR>HEPES>BF. NH₄Cl exposure is a well-known way to increase pH_i in all cells including corneal endothelium (Bonanno and Giasson, 1992a, c; Ogando et al., 2013; Roos and Boron, 1981). If increased pH_i stimulates glycolysis, then increasing pH_i by NH₄Cl incubation should increase lactate production. BCEC were washed with PBS and then incubated in BF or BR with or without 15 mM NH₄Cl all at pH 7.5, which increases steady-state pH_i by ~ 0.13 (Bonanno and Giasson, 1992a, c; Ogando et al., 2013; Roos and Boron, 1981). Figure 3C shows that lactate production was significantly increased in both BF and BR in the presence of NH₄Cl.

Laconic Lactate Sensor

Since Figure 3B shows that pH_i changes quickly upon changing the perfusing ringer, reaching a new steady-state within two minutes, and since PFK activity would be immediately affected by this change, we examined the continuous change in intracellular lactate [Lac_i] using endothelial cells transiently transfected with Laconic, a lactate sensitive FRET protein. Figure 4A shows a representative trace indicating an immediate increase in intracellular lactate when perfusion was switched from BF to BR. This was followed by a decrease in [Lac_i] back to the same starting value. Similar changes were observed when switching from BF to HEPES, however the rate, $1.5 \pm 0.8\%$ (F/F) per min, and peak change, $2 \pm 0.1\%$, in HEPES were significantly smaller (n=6, p<0.05) than when switching to BR, $10 \pm 1.2\%$ (F/F) per min and $9 \pm 1.5\%$ peak change, respectively. These results suggest that lactate production increased quickly as pH_i went up. Since there is zero lactate in the perfusing ringer, the driving force for lactate efflux is infinite, so this increased rate of production is likely matched by increased efflux bringing the [Lac_i] back to the same resting level.

If the rate of lactate production is increased in BR with the commensurate increased rate of efflux, then when applying a large pulse of lactate in the perfusing solution, the influx of lactate in BR will be met with greater resistance because of the greater baseline production rate and efflux. Whereas when the same lactate pulse is given in BF, where there is less baseline production and efflux, lactate influx will be easier. To test this prediction, we

perfused laconic transfected cells and applied a 10 mM pulse of lactate first in BF and then in HEPES (n=6) or BR (n=10). Figure 4B shows representative traces indicating that the rate of lactate influx and peak $[Lac_i]$ was greatest in BF, intermediate in HEPES, and least in BR, consistent with greater lactate production and efflux in BR. Paired differences of the initial rate and peak change between the response in BF and HEPES or between BF and BR were made and is summarized in Table 1.

Discussion

In this study we show that lactate production by corneal endothelial cells is increased in bicarbonate-rich conditions due to the increased pH_i in bicarbonate. Concomitant with increased lactate production is increased glucose consumption. Although the [glucose] used was low, the incubation conditions were identical and the rank order (BR>HEPES>BF) was consistent with lactate production. The increase in glycolytic flux was almost entirely blocked by GAPDH inhibition. An intermediate increase in lactate production and glucose consumption is observed in bicarbonate-free high HEPES ringer concomitant with an intermediate increase in pH_i . Independently, increasing pH_i in either BF or BR by exposure to NH_4Cl increased the rate of lactate production. There are no reports that NH_4Cl directly accelerates glycolytic activity, suggesting that it is the increased pH_i that is activating glycolysis. The HEPES and NH_4Cl results demonstrate that the increased glycolytic flux is not due to a secondary effect of CO_2/HCO_3^- , but rather are due to increased pH_i . Increasing pH_i by incubation in HEPES also increased oxygen consumption, consistent with increased glycolytic flux and an increased availability of pyruvate to enter mitochondria. Our results are consistent with a recent metabolomics study (Hamuro et al., 2020) and findings that phosphofructokinase (PFK), which catalyzes the conversion of fructose-6P to Fructose 1,6-bisphosphate, is the primary rate limiting enzyme of glycolysis and is sensitive to pH, where higher pH is activating (Kitajima et al., 1983; Trivedi and Danforth, 1966).

Altered capacity for lactate efflux or glucose uptake could also play a role. Efflux is by Lactate: H^+ cotransport, driven by the lactate and H^+ gradients. Increased pH_i in BR however, will decrease the driving force for lactate: H^+ efflux. In addition, we found that glucose uptake capacity as measured by 2-NBDG uptake was unaffected by BF, HEPES, or BR. Moreover, a change in expression of monocarboxylate transporters or glucose transporters (Glut) would not be expected within the very short time frame of these incubations. Glut1 is the predominant glucose transporter in corneal endothelium (Bildin et al., 2001; Ishida et al., 1995). If plasma membrane Glut1 expression or activity were increased, then 2-NBDG accumulation would also have increased. Therefore, we conclude that the increased lactate production is not due to an increase in lactate: H^+ cotransport activity or glucose transport capacity.

Real time changes in intracellular [lactate] (Lac_i) as measured by the FRET probe Laconic, provided additional insight to these events. $[Lac_i]$ increased transiently when perfusion was switched from BF to BR (or HEPES). An increase in $[Lac_i]$ could be due to increased production or decreased efflux of lactate. Since efflux is via lactate: H^+ cotransport, the increase in pH_i in BR (lower $[H^+]$) could slow efflux. However, if lactate production were unaffected and efflux was decreased, then the steady-state $[Lac_i]$ would remain elevated.

However, as shown in Figure 4 we see that $[\text{Lac}_i]$ decreased back to the starting level. Second, when a lactate pulse was introduced, $[\text{Lac}_i]$ increased less and more slowly in BR relative to BF, indicating that the influx of lactate from the pulse was fighting against an increased lactate efflux in BR. We conclude that in the presence of the high buffering capacity of HEPES or BR ringer, the increased steady-state pH_i activates glycolysis, most likely via PFK, increasing glycolytic flux and the production of lactate.

In the context of explaining the origin of the fluid transport (~60% of intact corneas) seen in cultured bovine corneal endothelial cells (Kuang et al., 2004; Narula et al., 1992), our findings support the lactate flux model for the endothelial pump. The model (Li et al., 2016; Li et al., 2020) states that lactate is taken up at the basolateral membrane of endothelial cells, where it creates a lower $[\text{lactate}]$ in the basolateral space. Intracellular lactate is then transported to the apical surface, where it creates a slightly higher $[\text{lactate}]$ in the apical unstirred layer. This creates a small osmotic gradient that then drives water flux from basolateral to apical. In the BCEC cultures, apical lactate efflux is greater than basolateral efflux, consistent with vectorial water flux linked to lactate. Lactate flux in cultured cells is also sensitive to the presence of bicarbonate and bicarbonate transport inhibition (Nguyen and Bonanno, 2011, 2012). Moreover, we can now add that the higher pH_i in endothelial cells incubated with high buffering capacity ringers like HEPES and BR, increases lactate production as well as efflux, thereby supporting the endothelial pump.

Lastly, whether pH_i is altered in endothelial dystrophies is unknown. However, in the mouse model of CHED where *Slc4a11* is knocked out, cultured endothelial cells have higher pH_i (Ogando et al., 2013; Zhang et al., 2017b). This is because *Slc4a11* supports H^+ influx driven by the membrane potential (Zhang et al., 2015). A metabolomics study indicated that glycolysis is preferred over glutaminolysis in *Slc4a11* KO cells (Zhang et al., 2017a; Zhang et al., 2017b), which suggests that lactate production is increased. Further studies are needed to determine if lactate production is altered in endothelial dystrophies and how this may contribute to corneal edema.

Acknowledgments

We would like to thank Edward Kim for expert technical assistance.

Funding Statement

This work was supported by NIH grant R01EY008834 (JAB). R.S is supported by a career starter grant from the Knights Templar Eye Foundation.

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Highlights

- Effect of Bicarbonate-free vs Bicarbonate-Rich Ringer on Corneal Endothelial Lactate Production
- Increased pHi in Bicarbonate
- Intracellular Lactate Measurement by Laconic
- Bicarbonate Rich Ringer Accelerates Lactate Production

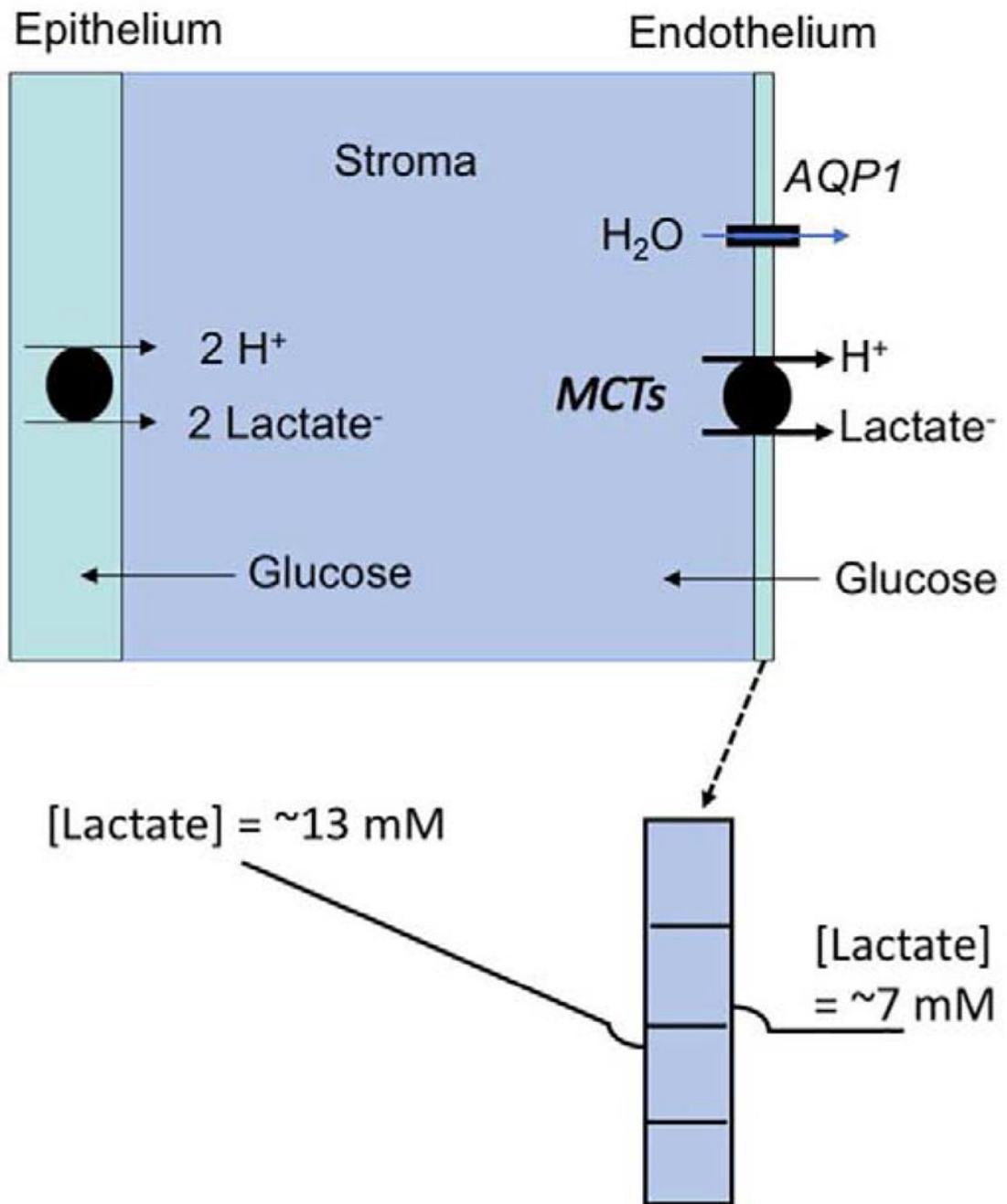


Figure 1.

Lactate- Water Flux Schematic Model. (Top) Glucose is taken up by corneal endothelial cells from the aqueous humor and diffuses into the stroma to the epithelium. Epithelial cells and stromal keratocytes produce 2 lactate molecules for every glucose metabolized in glycolysis. (Bottom) This creates a high [lactate] in the anterior cornea (average ~13 mM (Klyce, 1981)) relative to the aqueous (~7 mM) (Bergmanson et al., 1985). Therefore, a [lactate] gradient is formed decreasing from anterior cornea to endothelium, where monocarboxylate transporters (MCT1 and 4) take up lactate:H⁺ by secondary active transport,

creating a slight dip in [lactate] at the basolateral domain. Apical membrane MCT2 transfers lactate:H⁺ to the apical domain creating a slightly higher [lactate] in the apical unstirred layer. This transendothelial [lactate] gradient creates an osmotic driving force for water flux that is facilitated by aquaporin 1.

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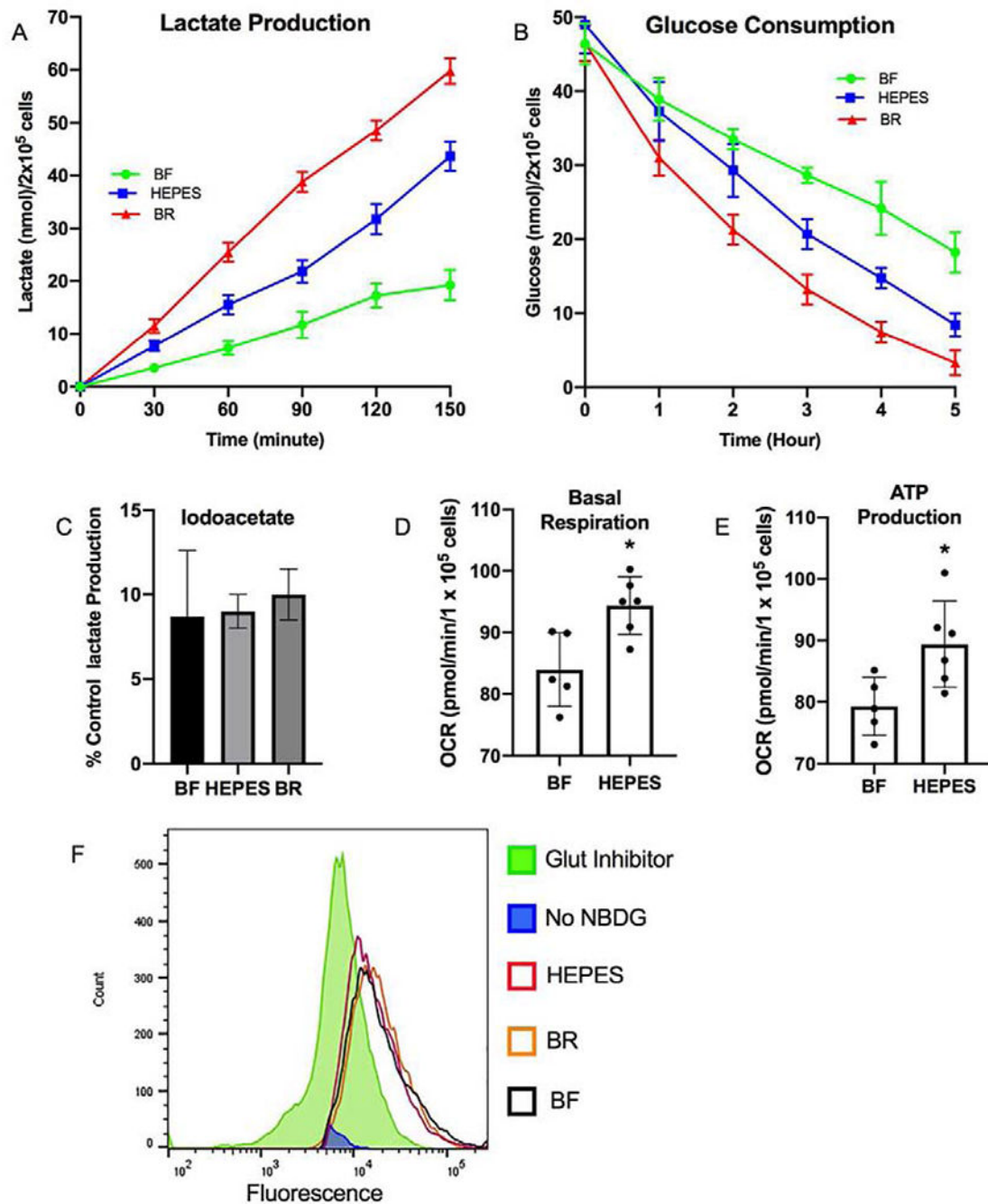
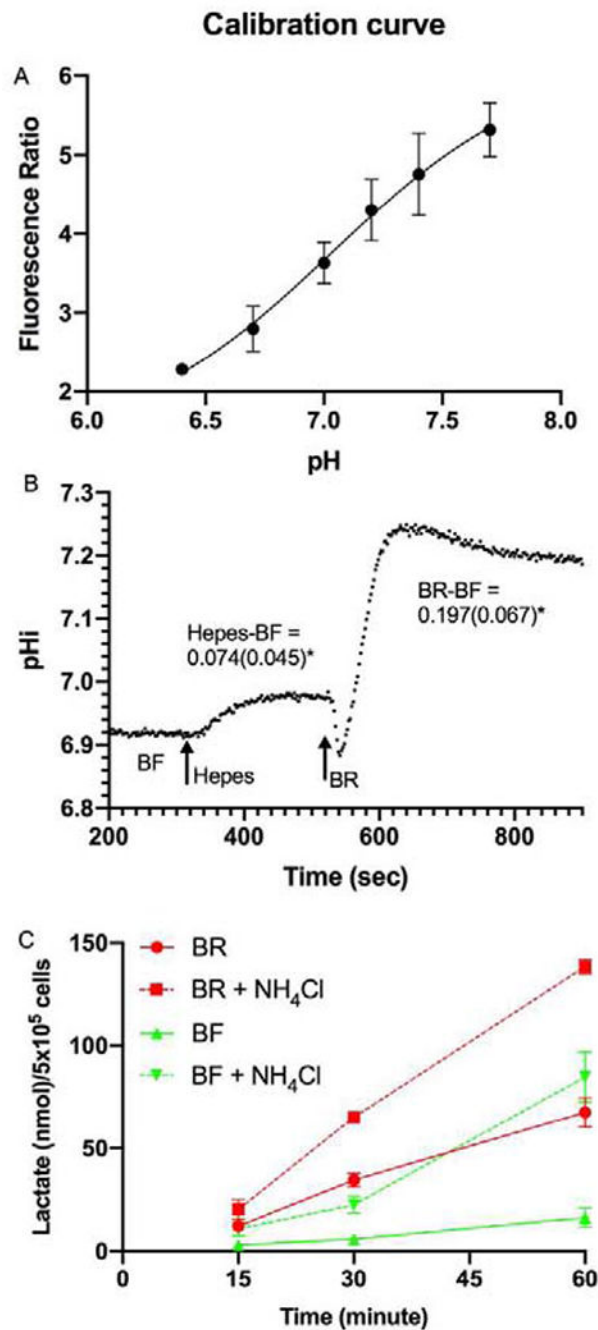


Figure 2. Effect of BF, HEPES, and BR Ringer on Glycolytic Activity in BCEC. A) Lactate production, $n=3$ per data point, *significantly different from BF, $p<0.05$. B) Glucose consumption. C) % of Control Lactate production at 2 hours in the presence of $100 \mu\text{M}$ iodoacetate, $n=3$ per condition. D) Oxygen Consumption Rate in BF ($n=5$) vs HEPES ($n=6$), * $p<0.05$. E) OCR related to ATP production. F) 2-NBDG uptake in BF, HEPES, and BR, $n=3$ per condition. No NBDG gate control and $20 \mu\text{M}$ Glut inhibitor WZB117 control are shown.

**Figure 3.**

Intracellular pH. A) Standard High [K] plus Nigericin (K^+/H^+ exchanger) calibration of fluorescence emission ratio (Ex_{495}/Ex_{440}) vs pH, $n=5$ per data point. B) Representative trace of pH_i of BCEC while perfused with BF, then HEPES, and then BR at arrows. Average (SD) of paired pH_i differences ($n=5$) in HEPES and BR vs BF is shown. C) Lactate production in BF and BR in the presence of 15 mM NH_4Cl , all at pH 7.5, $n=3$ per data point.

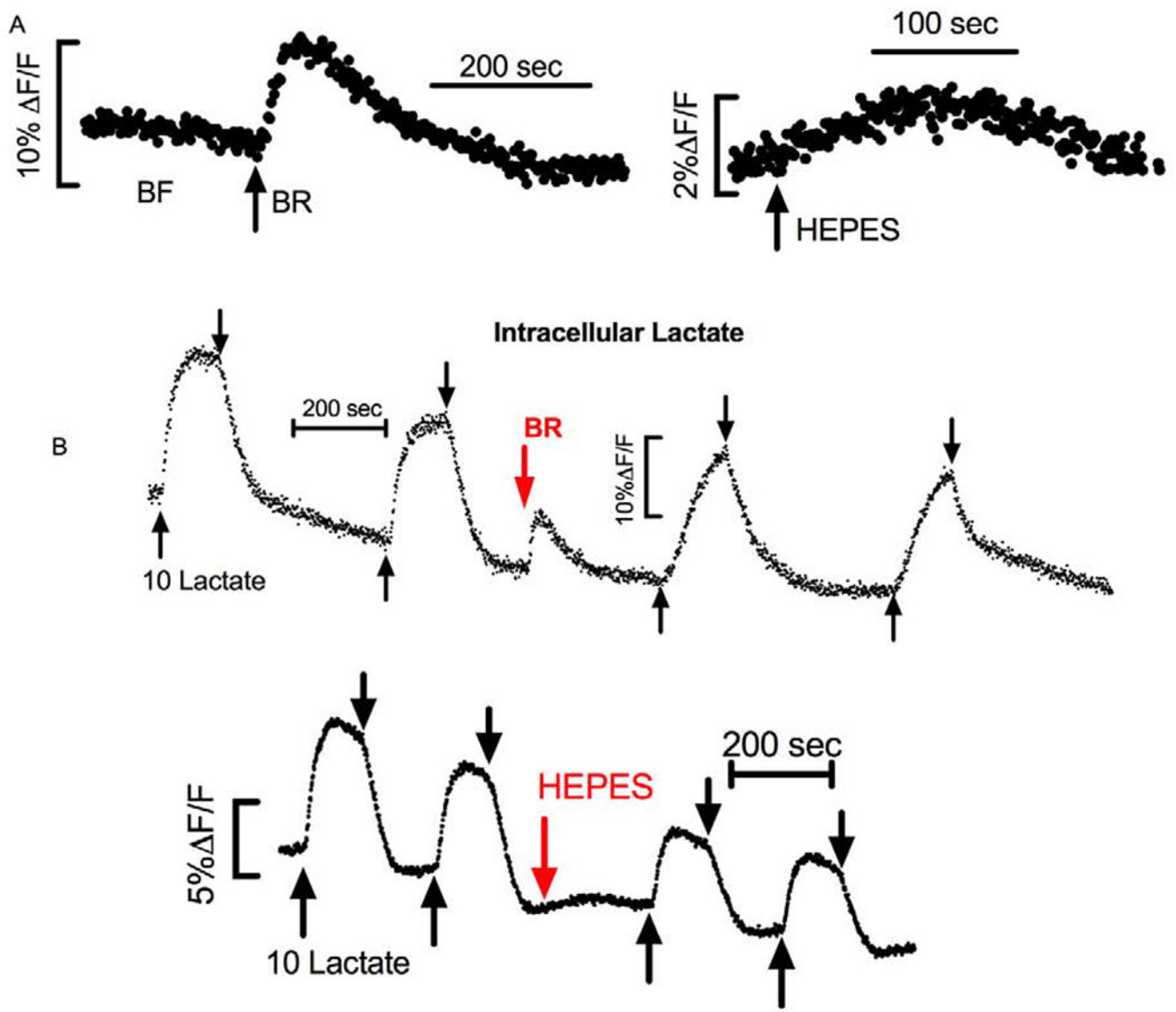


Figure 4. Relative Changes in Intracellular [Lactate] Measured with Laconic. A) Representative trace of Laconic transfected BCEC perfused with BF and then switched to BR at the arrow (Left) or switched to HEPES (right). B) Laconic transfected cells exposed to 2-minute pulse of 10 mM lactate (between up arrow and down arrow) in BF and then BR (top trace), and BF and then HEPES (bottom trace).

Table 1.

Rate and Peak Change in Laconic Fluorescence in Response to 10 mM Lactate Pulse Relative to BF

	% of Initial Rate	% of Peak
HEPES vs BF (n=6)	75.4±15 *	59.8±25.5 *
BR vs. BF (n=10)	19.5±8.8 *	37.6±22.5 *

*
 $p < 0.05$ paired *t*-test

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