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Research Paper

Glutaminolysis is Essential for Energy Production and Ion Transport in Human Corneal Endothelium

Wenlin Zhang [a,*], Hongde Li [b], Diego G. Ogando [a], Shimin Li [a], Matthew Feng [c], Francis W. Price Jr [c], Jason M. Tennessen [b], Joseph A. Bonanno [a,*]

[a] School of Optometry, Indiana University, Bloomington, IN 47405, USA
[b] Department of Biology, Indiana University, Bloomington, IN 47405, USA
[c] Price Vision Group, Indianapolis, IN 46260, USA

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ABSTRACT

Corneal endothelium (CE) is among the most metabolically active tissues in the body. This elevated metabolic rate helps the CE maintain corneal transparency by its ion and fluid transport properties, which when disrupted, leads to visual impairment. Here we demonstrate that glutamine catabolism (glutaminolysis) through TCA cycle generates a large fraction of the ATP needed to maintain CE function, and this glutaminolysis is severely disrupted in cells deficient in NH_3:H^+ cotransporter Solute Carrier Family 4 Member 11 (SLC4A11). Considering SLC4A11 mutations leads to corneal endothelial dystrophy and sensorineural deafness, our results indicate that SLC4A11-associated developmental and degenerative disorders result from altered glutamine catabolism. Overall, our results describe an important metabolic mechanism that provides CE cells with the energy required to maintain high level transport activity, reveal a direct link between glutamine metabolism and developmental and degenerative neuronal diseases, and suggest an approach for protecting the CE during ophthalmic surgeries.

1. Introduction

The Corneal Endothelium (CE) is a cell monolayer on the posterior surface of the cornea that is essential for maintaining corneal hydration, thickness, and transparency. CE is one of the most metabolically active tissues in the body with the highest mitochondrial density, second only to photoreceptors (Hogan et al., 1971), and corneal endothelial dystrophies are the most common indication for corneal transplants worldwide (Dorrepaal et al., 2007; Ghosheh et al., 2008). One major function of CE is to generate an outward osmotic driving force that counteracts an inward imbibition pressure generated by corneal stromal glycosaminoglycans (Barfort and Maurice, 1974). Maintenance of the osmotic force is dependent on primary active transport (Na^+-K^+-ATPase activity) and numerous secondary membrane ion transporters (Bonanno, 2012; Geroski and Edelhauser, 1984; Li et al., 2016). Together this is generally referred to as the CE pump function. Failure of the CE pump results in corneal swelling, loss of transparency, and in turn, visual impairment.

The robust ion and fluid transport activity of the CE imposes heavy metabolic demands on these cells (Bourne, 2003). Consistent with this, in addition to having a high mitochondrial density, CE dysfunction is a common symptom associated with mitochondrial disorders, such as Pearson Syndrome, Kearns-Sayre syndrome and Leigh's syndrome (Boonstra et al., 2002; Chang et al., 1994; Hayashi et al., 2000; Kasbekar et al., 2013). However, despite the intense energetic demands imposed by the CE pump function, the metabolic mechanisms required for maintaining high levels of energy remain largely unexplored. In this regard, understanding CE metabolism is of particular interest for cataract surgeries and corneal transplants, as CE pump function may be impaired for a time following these procedures, resulting in visually significant corneal edema. Currently, there is no way to clinically restore or augment CE pump function. Therefore, metabolic therapies that maximize available energy represent a potential means to improve CE pump function and surgical outcomes.

The membrane transporter *SLC4A11* has recently emerged as an important player in CE pump function. Not only is this gene highly expressed in the CE (Chng et al., 2013), but mutations in *SLC4A11* also cause a collection of developmental and/or degenerative corneal disorders such as Congenital Hereditary Endothelial Dystrophy (CHED), Harboyan Syndrome, Fuchs Endothelial Corneal Dystrophy (FECD), and Peters anomaly (Siddiqui et al., 2014; Vithana et al., 2006, 2008; Weh et al., 2014), as well as sensorineural deafness (Harboyan Syndrome,

* Corresponding authors.
E-mail addresses: wenlzhan@indiana.edu (W. Zhang), jbonanno@indiana.edu (J.A. Bonanno).

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CHED plus perceptive deafness) (Siddiqui et al., 2014). Intriguingly, SLC4A11 was recently found to function as an NH_3:$2H^+$ co-transporter (Zhang et al., 2015), indicating that ammonia metabolism plays an unexpected role in CE development, function, and degeneration. Since ammonia is a common waste product of amino acid catabolism, especially glutamine (Yang et al., 2009), these observations suggest that the CE may actively use glutamine to maintain Na^+-K^+-ATPase activity. In addition, the steady-state concentration of glutamine in human aqueous humor (0.6–0.8 mM) (Langford et al., 2007) is similar to plasma, further supporting the potential for glutamine metabolism.

Here we use rabbit, mouse, and human CE to investigate the link between CE metabolism and ammonia production. Strikingly, our findings reveal that the CE produces ammonia due to high levels of glutaminolysis. However, unlike stem cells and cancer cells that use glutamine to support biosynthesis (Le et al., 2012), we demonstrate that the non-proliferating CE uses glutamine to produce ATP for Na^+-K^+-ATPase activity to support physiological pump function. And this glutaminolysis is disrupted in SLC4A11 transporter deficient CE resulting in inhibition of pump function and significant pathology. This work sheds light on clinical metabolic therapies to facilitate CE function, pathogenesis of CHED, FECD and Harboyan syndrome, and suggest that the ammonia handling capacity offered by NH_3:H^+ transporter SLC4A11 is essential for cells actively metabolizing glutamine.

2. Materials and Methods

2.1. Animals

All mice were housed and maintained in pathogen-free conditions and used in the experiments in accordance with institutional guidelines and the current regulations of the National Institutes of Health, the United States Department of Health and Human Services, the United States Department of Agriculture and Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. RNA Extraction

Total RNA, from human corneal endothelial tissue, mouse endothelial tissue, immortalized HCEC cell line, and FECD patient corneal endothelial tissue were extracted and purified via RNeasy mini kit (#74104, Qiagen) with DNase digestion (#79254, Qiagen).

Healthy human corneal endothelium: Human donor cornea were obtained from Indiana Lion Eye Bank in 4 °C Optisol® GS medium. Corneal endothelium with Descemet's membrane was peeled off in Corneal Viewing Chamber (Stephens Instruments) using Submerged Cornea Using Backgrounds Away (SCUBA) technique. Peeled corneal endothelium sheet was processed immediately or stored in RNA*later*® (AM7020, Ambion) at 4 °C. Corneal endothelium sheet was rapidly frozen in liquid nitrogen and grinded followed by RNeasy column (Qiagen) purification.

Mouse corneal endothelium: Mouse cornea with sclera skirt was dissected from the globe. Corneal endothelium with Descemet's membrane was peeled off followed by RNA extraction similarly as described above.

FECD corneal endothelium: Patients diseased cornea endothelium samples were collected during Decrements Membrane Endothelial Keratoplasty (DMEK) surgery, and shipped on ice overnight in Optisol® GS medium. Corneal endothelium sheet was put into RNA*later*® in 4 °C till RNA extraction or processed immediately upon arrival. RNA extraction steps were the same as described above.

Immortalized HCEC cell line: HCEC cells were cultured in OptiMEM complete medium to confluent, RNA extraction steps were the same as described above.

2.3. PCR, qRT-PCR and Nested PCR

Complementary DNA was generated with High Capacity RNA-to-cDNA Kit (Applied Biosystems) at 10 ng RNA/μL reverse transcription. Sequences of human and mouse gene primers used are listed in Table S1 and Table S2, respectively. Conventional PCR was performed with MyCycler Thermal cycler (Bio-Rad) following the AmpliTaq® 360 DNA Polymerase protocol (Applied Biosystems). Real-time qPCR reactions were set up in triplicate using SYBR Green PCR Master Mix (Agilent Technologies). All assays used the same PCR conditions. A $2^{-\Delta\Delta Ct}$ experimental design was used for relative quantification and normalized to ACTB (mouse) or GAPDH (human) for differential expression levels of target genes. Nested PCR was conducted for genes with no CT value in real-time qPCR. An additional 40 cycles of PCR were conducted on reverse transcription PCR product using the same gene primers.

2.4. Immortalized HCEC Culture

Immortalized HCEC (Schmedt et al., 2012) were cultured at 37 °C, 5% CO_2 in appropriate plates or flasks coated with undiluted FNC Coating Mix® (AthenaES). Complete medium (OptiMEM-I®; Invitrogen) contains 8% FBS (Hyclone Laboratories Inc.), EGF 5 ng/mL (Millipore), pituitary extract 100 μg/mL (Hyclone Laboratories), calcium chloride 1.8 mM, 0.08% chondroitin sulfate (Sigma-Aldrich), gentamicin 50 μg/mL, and antibiotic/antimycotic solution diluted 1:100 (Invitrogen).

2.5. Immunofluorescence Staining

Excised mouse eyes or human donor cornea button were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4 °C overnight and paraffin embedded. Five-micrometer sections were then mounted on Super Frost slides (Fisher Scientific). The sections were de-paraffinized and hydrated in a graded ethanol series (100%, 95% and 70% and 50% ethanol and ddH_2O for 5 min each) and subject to antigen retrieval in 10 mM Na-citrate, then blocked with 2% BSA in PBS and incubated overnight at 4 °C with 1st antibodies. After washes in PBS, slides were incubated with Fluor-conjugated 2nd antibody for one hour and washed. Sections were mounted with prolong anti-fade mounting reagent with DAPI (Molecular Probes, Life Technologies) and imaged with AxioImager M1 microscope with AxioCam MRm camera (Zeiss).

The following antibodies were used: Rabbit polyclonal anti-Glutaminase (GLS1) antibody 1:200 (ab93434, Abcam); Rabbit anti-GLS2 antibody 1:200 (ab113509, Abcam); Mouse monoclonal anti-GGT1 antibody 1:200 (ab55138, Abcam); Rabbit anti-ZO1 1:200 (402200, Life Technologies); Mouse anti-ZO1 1:200 (339100, Invitrogen); Rabbit anti-Nitrotyrosine 1:200 (A-21285, Thermo Scientific); Secondary Alexa-488 and Alexa-568 antibodies (Molecular Probes) were used at 1:200 concentrations. Mean intensity (MFI) quantification of endothelium was conducted using ImageJ.

2.6. Hematoxylin and Eosin (H&E) Staining

Deparaffinized sections (5 μm) were stained with Hematoxylin and Eosin (H&E), and imaged with AxioImager M1 (Zeiss).

2.7. Alizarin Red Staining of Corneal Endothelium

As previously described (Park et al., 2012), rabbit and/or human cornea button was briefly immersed and rinsed with pH 4.2 0.9% saline, then stained with pH 4.2 0.2% Alizarin Red 0.9% saline for 2 min, and rinsed with pH 7.2 0.9% saline.