Lithium treatment increases endothelial cell survival and autophagy in a mouse model of Fuchs endothelial corneal dystrophy

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ABSTRACT

Background Lithium previously has been shown to reduce both endoplasmic reticulum (ER) and oxidative stress in other in vitro and in vivo model systems. We investigated lithium’s effects on cultured corneal endothelial cells (CECs) exposed to these types of stress and in a mouse model of Fuchs endothelial corneal dystrophy (FECD).

Methods Viability of cultured bovine CECs was determined by CellTiter-Glo. 2-month-old Col8a2(Q455K) and C57/Bl6 wild type animals were divided into two groups of 15 mice. Group I received 0.2% lithium carbonate-containing chow and Group II received control chow for 7 months. Confocal microscopy, transmission electron microscopy, real-time PCR (RT-PCR) and western blot were performed.

Results Pretreatment with lithium increased viability of cultured CECs after H2O2 and thapsigargin exposure compared with untreated controls (p<0.05). In vivo analysis of mouse corneal endothelium showed the following: endothelial cell density of lithium treated Q455K was higher than for untreated Q455K (p<0.01). Transmission electron microscopy of lithium treated Q455K showed normal endothelium with enlarged autophagosomes, but untreated Q455K showed dilated ER and guttae. Compared with untreated Q455K endothelium, lithium treated Q455K showed significant upregulation of P62, Tmem74, Tmem91f1 and Tmem166 by RT-PCR and of Atg5-12 conjugate by western blotting indicating that lithium treatment increased autophagy. Although RT-PCR unexpectedly showed increased levels of lithium response genes, caspase 12, Gsk3β, Arfβ2 and Impα1, western blotting showed the expected downregulation of Arfβ2 and Impα1 proteins in response to lithium treatment.

Conclusions Lithium increases cultured CEC survival against ER and oxidative stress. Increased autophagy in lithium treated endothelium in a mouse model of FECD suggests autophagy may contribute to increased endothelial cell survival.

INTRODUCTION

Fuchs endothelial corneal dystrophy (FECD) is characterised by progressive loss of corneal endothelial cells, alterations in the extracellular matrix and loss of vision from corneal clouding.1 FECD affects upwards of 4% of the US population over the age of 40.2 Currently no definitive non-surgical treatments exist for FECD, and this disorder is a leading cause of corneal transplantation accounting for upwards of 23% of cases.3 Mutations in multiple genes have been associated with FECD (for a complete list see http://www.ncbi.nlm.nih.gov/omim/136800) including a missense mutation resulting in a glutamine to lysine substitution at amino acid 455 (Q455K) in the α 2 collagen VIII (COL8A2) gene causing an early onset form of the disease4 and a more common non-coding single nucleotide polymorphism in the transcription factor 4 (TCF4) gene linked to late onset disease.5 Endoplasmic reticulum (ER) stress as shown by our previous work1 and oxidative stress as shown by others6 play important roles in the cellular pathogenesis of FECD. We have described previously a Q455K Col8a2 transgenic knock-in mouse model of FECD which showed clinical features strikingly similar to human patients and also demonstrated endothelial cell ER stress and apoptosis, thereby confirming the role of ER stress in both early and late onset FECD.5 7-9 Lithium has a wide range of protective cellular effects against a variety of stressors.8 Lithium reduces ER stress-mediated apoptosis via inhibition of caspase-12 and cleavage of caspase-3 in the ER.5 Lithium treatment can decrease the superoxide dismutase to catalase ratio significantly, which is associated with decreased oxidative stress by lowering hydrogen peroxide levels.8 Protracted lithium pre-treatment is cytoprotective against thapsigargin-induced cytotoxicity resulting from ER stress in PC12 cells.10 Lithium induces autophagy by way of the PI3K signalling pathway and has been proposed as a treatment for neurodegenerative diseases such as Huntington’s disease.11 Lithium overcomes the autophagic defect in CbCln3(Δex7/8/Δex7/8) cerebellar cells and increases their viability under conditions of IMPase inhibition.12 Given the protective effects of lithium against ER and oxidative stress, we sought to investigate its effects on cultured corneal endothelial cells exposed to these types of stress and on in vivo corneal endothelium in a mouse model of FECD.

METHODS

Cell culture

Bovine corneal endothelial cells were scraped from the excised corneas of freshly enucleated globes with a surgical blade, and primary cultures were established by resuspending cells in Dulbecco’s modification of Eagle’s minimum essential medium (Cellgro, Manassas, Virginia, USA) supplemented with 10% fetal calf serum, antibiotic antimycotic solution (10 000 units of penicillin (base), 10 000 μg of streptomycin (base) and 25 μg of amphotericin B/mL) (Invitrogen, Grand Island, New York, USA) and 50 μg/mL of gentamicin (Invitrogen). Cells were grown at 37°C in 5% CO2 in air on a 6-well plate (Cytoone, Orlando, Florida,
USA). Once confluence was reached (usually 7–10 days), the primary cultures were subcultured to 96-well plates (Cytoone) in the same medium.

**Cell treatment with lithium**

Lithium carbonate (Sigma, St Louis, Missouri, USA) was freshly dissolved in phosphate-buffered saline (Gibco-BRL, Carlsbad, California, USA) at room temperature before use. To examine the effects of lithium on corneal endothelial survival, corneal endothelial cells were incubated in quadruplicate wells of a 96-well plate at several lithium concentrations (0–1000 µM) for 48 h. After pretreatment with lithium for 48 h, the cultures were then treated with 100 µl medium containing thapsigargin (28 µM) for 24 h or H₂O₂ (0.6 mM) for 2 h at 37°C in a humidified 5% CO₂ atmosphere. Negative control (no lithium treatment and no H₂O₂ or thapsigargin conditioning) was also included to determine baseline cell viability. At the end of incubation, cell viability was determined by adding 100 µl of CellTiter-Glo luminescent reagent (Promega, Madison, Wisconsin, USA) which produces light in direct proportion to the amount of ATP and viable cells present. The luminescence was measured using a FLUO star OPTIMA spectrophotometer (Optima, Tokyo, Japan). The cell viability (%) related to control was calculated by 100× luminescence in H₂O₂ or thapsigargin conditioned cells/luminescence in the negative control (no lithium and no H₂O₂ or thapsigargin).

**Animals**

Weaned 2-month-old mice homozygous for the Col8a2 Q455K mutation and 2-month-old wild type (WT) C57/B6 mice (Jackson Laboratory, Bar Harbor, Maine, USA) were divided into two groups of 30 mice for each treatment. Corneal endothelium from mutant mice at 2 months of age shows no differences with WT mice on confocal microscopy (unpublished data). Group I (lithium group, 15 mutant and 15 WT for a total of 30 mice) received 0.2% (weight/weight) lithium carbonate–thapsigargin. Group II (control group, 15 mutant and 15 WT for a total of 30 mice) received control rodent chow and pure drinking water for 7 months. All mice were housed in the Johns Hopkins Oncology Resources Animal Facility in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. The study protocols described herein were reviewed and approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

**Confocal microscopy**

All mice were euthanised by isoflurane (Vedco Inc., St Joseph, Missouri, USA), which was verified by checking for the absence of respirations followed by cervical dislocation. Immediately after euthanasia, one eye of each mouse was randomly selected for examination by confocal microscopy (Nidek Confoscan 3, Fremont, California, USA). Endothelial cell imaging and quantitative analyses were performed using the Confoscan 3 software.

**Transmission electron microscopy**

Both eyes of two mice (four eyes total) randomly selected from each group were analysed. After confocal microscopic examination, whole globes were enucleated and fixed in 2.5% glutaraldehyde/2% paraformaldehyde, pH 7.4, overnight at 4°C. Corneas were excised and bisected. Corneal halves were then washed in 0.1 M phosphate buffer followed by a 2 h, room temperature fixation in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4. The specimens were then washed and dehydrated in a series of 50%–100% ethanol followed by a 1 h fixation in 1% uranyl acetate in 100% ethanol at room temperature in the dark. Postdehydration, specimens were washed twice in propylene oxide for 15 min each and incubated in a 1:1 mixture of propylene oxide to LX-112 resin (Ladd Research, Williston, Vermont, USA) overnight at room temperature. Specimens were submerged in 100% resin, subjected to vacuum for 5 h before embedding in freshly made LX-112 resin, and polymerised at 60°C for 2 days before sectioning. A total of 68 nanometer ultrathin sections were cut with a Leica Ultramicrotome UCT (Wetzlar, Germany) and imaged using a Hitachi H7600 transmission electron microscope (Pleasanton, California, USA).

**Real-time PCR**

After confocal microscopic examination, a total of five mice were randomly selected from each group. Both eyes of each mouse were extracted, Descemet membranes (DMs) were stripped with forceps, and both DMs containing endothelium from a single mouse were pooled as a single sample (n). Five mice (n=5) were used for each group. Total RNA was extracted from murine corneal endothelium using TRIzol reagent (Invitrogen) followed by RNeasy column (Qiagen, Hilden, Germany) purification. Complementary DNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). The resulting complementary DNA (3 µl cDNA) was preamplified with TaqMan PreAmp MasterMix and mouse specific primers (PCR primers for the examined genes are listed in table 1). RT-PCR was performed using the TaqMan Gene Expression Master Mix (2×) (Applied Biosystems), preamplified cDNA products (diluted 1:20; 5 µl), nuclease free water and mouse specific primers in a 20 µl reaction volume. The relative gene expression in Col8a2Q455K/Q455K and WT endothelium was normalised to β-actin. A no-template control was included in each quantitative RT-PCR experiment to confirm the absence of DNA contamination. All assays used similar amplification

**Table 1**

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*Applied Biosystems, Foster City, California, USA.
efficiency, and a ΔCₜ experimental design was used for relative quantification. Data analysis was performed using StepOne software (V2.2, Applied Biosystems). Statistical analysis of quantitative RT-PCR data between the groups was performed using analysis of variance (ANOVA) and DataAssist Software V3.0 (Applied Biosystems). A p value<0.05 was considered statistically significant.

**Western blotting**

After confocal microscopic examination, a total of four mice were randomly selected from each group. Both eyes of each mouse were extracted, DMs were stripped with forceps and both DMs containing endothelium from a single mouse were pooled as a single sample (n). Four mice (n=4) were used for each group. DM and endothelial cells were homogenised in Tissue Protein Extract Reagent (Thermo Fisher Scientific, Rockford, Illinois, USA) with 1% protease inhibitor cocktail (Sigma) and 1% ethylenediaminetetraacetic acid (Sigma). The mixture was then microcentrifuged for 10 min at 12 000 rpm. The lysate was removed and the protein concentration was quantified by BCA Protein Assay Kit (Thermo Fisher Scientific). An amount of 8 μg of protein were mixed with 10 μL of 4X loading dye (NuPage, Invitrogen, Carlsbad, California, USA) with 2-mercaptoethanol (Sigma) and heated at 65°C for 5 min.

Samples were loaded onto a 10% Tris–HCl Ready Gel (BioRad, Hercules, California, USA) and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis separation for 1 h at 120 V. Proteins were transferred to a polyvinylidene fluoride membrane (BioRad) and incubated in blocking buffer for 1 h at room temperature. Loading controls were assayed by probing with β-actin (1 : 1000, Cell Signaling Technology) as primary antibody after stripping with Restore Stripping Buffer (Thermo Fisher Scientific). Low abundance proteins were detected using SuperSignal West Dura (Thermo Fisher Scientific) and higher abundance proteins were detected using HyGlo Quick Spray (Denville Scientific, Metuchen, New Jersey, USA). Densitometry analysis was performed using Image J as previously described (http://www.lukemiller.org/journal/2007/08/ quantifying-western-blots-without.html).

**Transmission electron microscopy**

Lithium treated Q455K corneal endothelium showed a combination of normal appearing cells with compact organellae (figure 2A) and cells with grossly enlarged autophagosomes containing remnants of degraded organellae (figure 2B). Untreated, control Q455K endothelium showed severely dilated ER membranes and increased variation in cell size and shape. Endothelium from lithium treated and untreated WT mice showed a normal phenotype with no guttae and a characteristic monolayer of hexagonal cells with regularly spaced size and shape (figure 1B). Corneal endothelial CD of lithium treated Q455K mice (1847 ± 227 cells/mm²) was 25% higher than for untreated Q455K mice (1472 ± 83) (p<0.01, figure 1C). CD of untreated, control WT mice (2207 ± 133 cells/mm²) was significantly higher than both lithium treated and untreated Q455K mice (figure 1C). Percent hexagonal cells, an additional marker of CEC integrity, was 22% higher in lithium treated Q455K mice (32.6% ± 7.6%) and 38% higher in untreated WT mice (37.0% ± 9.4%) compared with untreated Q455K mice (26.8% ± 8.25) (p<0.01 for both, figure 1C).
Finally, we sought to confirm cellular effects of lithium by assaying expression of the following genes associated with downregulation by lithium treatment. Surprisingly, significant upregulation of Caspase 12 (1.65±0.02-fold), Gsk3β (1.52±0.21-fold), Arrβ2 (1.61±0.17-fold) and Impa1 (1.35±0.18-fold) were found by RT-PCR in Q455K-Lithium versus untreated Q455K-Control endothelium (figure 3C). However, western blotting showed the expected downregulation of Impa1 (0.68±0.14-fold) and Arrβ2 (0.41±0.22-fold) in Q455K-Lithium versus untreated Q455K-Control endothelium (figure 3C).

DISCUSSION

FECD is characterised by progressive loss of corneal endothelial cells and is a leading cause of corneal transplant surgery. ER and oxidative stress contribute to the pathogenesis of FECD. Lithium prevents ER and oxidative stress-mediated apoptosis via a variety of cellular effects, and we found that lithium increases survival in cultured corneal endothelial cells exposed to ER and oxidative stress. Although highly reproducible, the relatively modest increase in survival in lithium treated versus untreated cultured cells may reflect the higher intensity and shorter duration cell stress used in the culture system compared with presumably lower levels and longer duration cell stress in vivo.

Given others’ work and our initial results in cultured corneal endothelial cells indicating the protective effects of lithium against ER and oxidative stress, we sought to evaluate the effects of chronic, oral, low-dose lithium treatment on the corneal endothelium in vivo using our knock-in mouse model of FECD homozygous for the Q455K Col8a2 mutation. In clinical confocal images of 9-month-old animals, endothelium of lithium treated Q455K mice showed mild endothelial guttae highly characteristic of FECD, but endothelium from untreated Q455K mice showed multiple guttae and increased variation in cell size and shape. Corneal endothelial CD and percent hexagonal cells of lithium treated Q455K mice were higher than for untreated Q455K mice.

Figure 1  (A) Cell viability of lithium treated bovine corneal endothelial cells after H2O2 or thapsigargin conditioning increased compared with untreated control (*p<0.05). Data represent the mean±SD. (B) Confocal images of corneal endothelial cells in homozygous Q455K mutant mice treated with lithium (Q455K-Lithium, representative images from two animals shown) have fewer guttae (white arrows) and relatively normal cell size and shape compared with untreated homozygous Q455K (Q455K-Control, representative images from two animals shown) mice. Wild type mice treated (WT-Lithium) or untreated (WT-Control) with lithium show normal endothelium (scale bar=60 μM for all panels). (C) Scatter plot shows higher corneal endothelial cell density in Q455K-Lithium mice compared with untreated Q455K-Control mice. Untreated WT-Control mice are shown for comparison. Per cent hexagonality of corneal endothelial cells in Q455K-Lithium mice is higher than Q455K-Control group (*p<0.01 compared with Q455K-Control). Horizontal bars=mean.
Based on the positive in vivo effects of lithium on endothelial CD and morphology, we performed transmission electron microscopy to evaluate its effects on the ultrastructure of corneal endothelial cells. Lithium treated Q455K corneal endothelium showed normal appearing cells interspersed with cells containing grossly enlarged autophagosomes, whereas untreated
Q455K endothelium showed severely dilated ER and guttae. We hypothesised that increased autophagy induced by lithium might enhance cell survival in Q455K endothelium exposed to ER stress. Thus, we next confirmed increased expression of autophagy markers in the endothelium of lithium treated Q455K mice compared with untreated control Q455K mice. Finally, we sought to confirm the pharmacological effects of lithium on the corneal endothelium of treated animals by assessing the expression of caspase 12, glycogen synthase kinase 3β (Gsk3β), arrestin 2β (Arr2β) and Impa1.16 Somewhat surprisingly, lithium caused upregulation of caspase 12, Gsk3β, Arr2β and Impa1 by RT-PCR, whereas western blotting showed the expected downregulation of Arr2β and Impa1. Chronic lithium treatment has been shown to upregulate Impa1 mRNA levels in mouse hippocampus which may reflect a compensatory response to inositol depletion.17 A similar phenomenon could explain the apparent inconsistency between mRNA and protein levels in our selected lithium response genes.

Induction of autophagy by lithium is neuroprotective and is associated with inhibition of IMPA.11 12 Autophagy is a complex cellular process which occurs in response to ER stress and oxidative stress as a mechanism to enhance cell survival.18 19 Given the role of ER stress in FECD,5 7 it is provocative to suggest that the endothelial cell survival effect of lithium described here is primarily mediated through increased autophagy. However, other possibilities cannot be excluded such as lithium’s antiapoptotic effects resulting from inhibition of glycogen synthase kinase 3.16

In conclusion, our results reveal that lithium can increase corneal endothelial survival in a cell culture and mouse model of FECD and thus represent the first in vivo evidence of a potential medical treatment for FECD. Our findings further suggest that increasing autophagy through other agents in addition to lithium may be a rational therapeutic approach. As mutations in multiple genes (COL8A2, SLC4A11, TCF4, TCF8, LOXHD1 and CLU/TGFB1) have been associated with causing FECD,2 4 20 24 additional work will be needed to confirm whether lithium and increased autophagy can improve the disease course associated with other genetic causes of FECD. As previous reports demonstrating the role of ER and oxidative stress in FECD used genetically undifferentiated (ie, unknown genotypes), late stage disease tissue obtained at corneal transplantation,5 6 we speculate that lithium’s cytoprotective effects against these processes could make it a generalised ‘endothelial survival factor’ with broad therapeutic effects in FECD. Future investigations of lithium as a treatment for FECD could involve clinical trials to assess its efficacy in patients with different genetic causes of FECD as well as to assess for adverse effects of treatment which are well documented with lithium’s use in psychiatric disease. However, the use of topical or local routes of ocular administration, if technically feasible, could be attractive approaches to mitigate the latter issue.

Contributors All authors were involved in study design; data collection; data management and analysis; and data interpretation, preparation of the initial draft of manuscript, review and final approval of the manuscript. AI takes overall responsibility for this work.

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REFERENCES