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<th>Activation of the NFAT–calcium signaling pathway in human lamina cribrosa cells in glaucoma</th>
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<tr>
<td>Publication date</td>
<td>2018</td>
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<tr>
<td>Type of publication</td>
<td>Article (peer-reviewed)</td>
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[http://dx.doi.org/10.1167/iovs.17-22531](http://dx.doi.org/10.1167/iovs.17-22531) |
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Glaucoma is the most common cause of treatable visual impairment in the developed world. It is a chronic neurodegenerative disease characterized by cupping and pallor of the optic nerve head (ONH) and by the slow progressive death of retinal ganglion cells. Histologically, there is remodeling of extracellular matrix (ECM) in the lamina cribrosa (LC) region leading to fibrosis of the LC connective tissue, which is driven in part by profibrotic growth factors such as oxidative stress, mechanical stretch, and growth factors cause fibroblasts to change their phenotype and differentiate into myofibroblasts. The main features of myofibroblast differentiation are the disproportionate increase in the expression of structural ECM proteins, matricellular proteins, smooth muscle α-actin (αSMA), and TGF-β. LC cells have many characteristics of myofibroblasts including the expression of α-SMA, and a marked expression of profibrotic genes and proteins (collagen 1α1 [Col1A1], periostin, fibronectin) upon stimulation with cyclic stretch, oxidative stress, and endothelin. In physiological wound healing, the activity of myofibroblasts is terminated when the tissue is repaired and they later disappear from the wound by apoptosis. In pathologic wound healing,
However, myofibroblast activity (associated with a lack of apoptosis) persists and leads to deposition of excessive ECM components in which normal tissue is replaced by permanent fibrotic scar tissue, such as that seen in the LC in glaucoma. Chronic elevation of intracellular calcium ([Ca$^{2+}$]) levels activates downstream Ca$^{2+}$-dependent signaling pathways that can mediate maladaptive ECM remodeling often leading to fibrosis. For example, in the heart, one such pathway is regulated by the Ca$^{2+}$-calmodulin-dependent phosphorylase calcineurin, which has been shown to be crucial for pathologic cardiac hypertrophy and fibrosis. Increased [Ca$^{2+}$], activates a serine/threonine phosphatase, calcineurin, that binds to Ca$^{2+}$ and calmodulin, leading to dephosphorylation of the transcription factor, nuclear factor of activated T-cells (NFAT) in the cytoplasm. Dephosphorylated NFAT translocates into the nucleus where it complexes with other transcription factors to regulate and drive hypertrophic gene transcription, thus providing a direct link between [Ca$^{2+}$], signaling and gene expression. Cyclosporin A (CsA) is a calcineurin inhibitor that blocks NFAT dephosphorylation in the cytoplasm, precluding its movement to the nucleus and thereby preventing increased gene transcription levels of profibrotic ECM gene production. In addition, when Ca$^{2+}$ entry is blocked or calcineurin activity is inhibited, NFAT is rephosphorylated by NFAT kinases and rapidly leaves the nucleus, and NFAT-dependent gene expression is terminated. CsA is an immunosuppressive and antifibrotic agent used to treat a variety of systemic immunosuppressive conditions including rheumatoid arthritis and also for ocular inflammation, where it has been shown to inhibit fibrosis and inflammatory cell infiltration in conjunctivitis, and in dry eye treatment.

We have previously shown elevated baseline and hypotonic cell membrane stretch-induced increase in [Ca$^{2+}$], in glaucoma LC cells. Our experimental results, using different pharmacologic Ca$^{2+}$ inhibitors (verapamil [L-type Ca$^{2+}$ channel blocker] and 2-APB [store-operated Ca$^{2+}$ channel blocker]), showed that glaucoma LC cells also have higher baseline levels of Ca$^{2+}$-dependent potassium ( maxi-K) ion activity. Our results also showed that both sources of cytosolic Ca$^{2+}$ elevation (Ca$^{2+}$ entry and release from intracellular stores) contribute to the abnormally elevated [Ca$^{2+}$], in glaucoma LC cells. Also, the maxi-K activity was sensitive to stretch as gadolinium (stretch-activated Ca$^{2+}$ entry channel blocker) significantly reduced the hypotonic stretch-induced increase in maxi-K activity in normal LC cells. In addition, we found that glaucoma LC cells have markers of oxidative stress and mitochondrial dysfunction, and express raised levels of several ECM genes such as periostin, collagen 1A1, and TGF-β1 transcription in normal and glaucoma LC cells. These changes in RPE cells and trabecular meshwork (TM) cells contribute to the abnormal elevation of [Ca$^{2+}$], in glaucoma LC cells. Also, the cytotoxic effect of CsA was tested and no effect was observed in normal and glaucoma LC cells were cultured in DMEM containing CsA (10 µM) overnight before being harvested for experiments. CsA was dissolved in vehicle control ethanol and at the matched concentration (10 µM); the vehicle control had no effect on either NFATc3 protein expression or its dephosphorylation or profibrotic gene transcription. Dose-response experiments under both hypotonic cell membrane stretch and oxidative stress effects on LC cell viability have been performed, and the hypo-osmolality (232 mOsm) and H$_2$O$_2$ (100 µM) used had no effect on cell viability. Also, the cytotoxic effect of CsA was tested and no effect was observed on LC cell viability at the concentration used (10 µM).

### Hypotonic Cell Stretch and Hydrogen Peroxide Treatments of Lamina Cribrosa Cells

We investigated the effect of two glaucoma-related stimuli (hypotonic cell membrane stretch and oxidative stress) on [Ca$^{2+}$], and downstream profibrotic ECM gene transcription. Hypotonic cell membrane stretch involved rapid replacement of the physiological isotonic solution (osmolality: 323 ± 6 mOsm) consisting of (mM): 120 NaCl, 6 KCl, 1 MgCl$_2$, 2 C$_6$H$_5$O$_7$, 5.4 HEPES, and 80 D-mannitol with hypotonic solution (osmolality: 232 ± 8 mOsm), which was prepared by omitting D-mannitol from the isotonic solution. Oxidative stress involved hydrogen peroxide (H$_2$O$_2$, 100 µM) incubation for 6 hours, looking at the effects on NFATc3 and profibrotic genes such as periostin, Col1A1, and TGF-β1 transcription in normal and glaucoma LC cells. To evaluate the effect of CsA on profibrotic gene transcription and NFATc3 expression, both normal and glaucoma LC cells were cultured in DMEM containing CsA (10 µM) overnight before being harvested for experiments. CsA was dissolved in vehicle control ethanol and at the matched concentration (10 µM); the vehicle control had no effect on either NFATc3 protein expression or its dephosphorylation or profibrotic gene transcription. Dose-response experiments under both hypotonic cell membrane stretch and oxidative stress effects on LC cell viability have been performed, and the hypo-osmolality (232 mOsm) and H$_2$O$_2$ (100 µM) used had no effect on cell viability. Also, the cytotoxic effect of CsA was tested and no effect was observed on LC cell viability at the concentration used (10 µM).

### Calcium Imaging

The dual-wavelength ratiometric Ca$^{2+}$ imaging technique, using the membrane-permeable ester of the Ca$^{2+}$-sensitive fluorescent dye Fura-2-AM to measure [Ca$^{2+}$], is a standard technique extensively used by numerous laboratory groups worldwide mostly based on the work by Grynkiewicz et al. In the eye, this protocol has been used to measure changes in RPE cells and trabecular meshwork (TM) cells. We previously used Fura-2-AM to record the real-time changes in LC cultured cells subjected to hypotonic and cyclic stretch. In brief, confluent LC cells were loaded with 5 µM Fura-2-AM for 45 minutes in darkness at room temperature, washed twice, and kept in Ca$^{2+}$-free physiological isotonic solution (osmolality was at 323 ± 6 mOsm), and then the coverslip was mounted on the stage of an inverted epifluorescence microscope (Diaphot 200; Nikon, Tokyo, Japan). A monochromator dual-wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm with an Okra Imaging camera.
(Hamamatsu, Japan). The images of multiple cells collected at each excitation wavelength were processed using Openlab2 software (Improvement system, London, UK) to provide ratios of Fura-2-AM fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380). All experiments were corrected for background fluorescence before the Fura-2-AM ratio was calculated. The [Ca²⁺]ᵢ, was obtained according to the equation provided by Grynkiewicz et al.⁵⁵:

\[
[\text{Ca}^2{}^+{}] = \frac{R}{R_\text{min}} = \frac{F_{\text{380 Ca-free}} \cdot (R_\text{max} - R_\text{min})}{F_{\text{380 Ca-free}} \cdot (R_\text{max} - R_\text{min})}
\]

where the dissociation constant (Kᵣ) was assumed to be 225 nM, R₀ is the ratio of fluorescence measured at 340 nm (F₃₄₀) over 380 nm (F₃₈₀) in a “nominally” Ca²⁺-free solution, Rₘₐₓ is the ratio of fluorescence measured at 340 nm over 380 nm.

Fluorescence traces shown represent [Ca²⁺]ᵢ, values that are representative of at least 50 to 80 cells and are presented as [Ca²⁺]ᵢ, fluorescence ratio (340/380). The histograms (average data from seven age-matched normal eye donors and seven glaucoma patients) are presented as the absolute changes in [Ca²⁺]ᵢ, during the experiments, which typically take 20 minutes, the pH and temperature are continuously monitored and maintained constant.

To study the release of Ca²⁺ from internal stores, the LC cells were washed twice and kept in Ca²⁺-free isotic solution, and ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA) (5 mM) was added to remove the residual external Ca²⁺. Thapsigargin (TG) (1 μM) was added to block the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) pumps, inducing a complete depletion of Ca²⁺ from intracellular stores. The isotic solution was then replaced with hypotonic solution, and CaCl₂ (2 mM) was added extracellularly to facilitate Ca²⁺ influx via the store-operated channels that opened because of Ca²⁺ store depletion.

**Cell Lysate Preparation and Western Blot Analysis**

Normal and glaucoma LC cells were plated in T75-mm tissue culture dishes and grown until 90% confluent and placed in serum-free medium for 24 hours. The serum-free medium was then removed and cells were subjected to oxidative stress using H₂O₂ for 6 hours at 37°C with or without CsA treatment. The reaction was stopped by removal of the medium and introduction of ice-cold phosphate-buffered saline (PBS). The crude cell lysate was collected using radio immuno-precipitation assay (RIPA) buffer containing protease inhibitor cocktail, and then the cells were incubated on ice for 5 minutes and subsequently centrifuged for 15 minutes at 17,968 x g at 4°C. The cleared supernatant was collected and the protein concentration was quantified using the Bradford method. Protein extracts (20 μg) were electrophoresed on 10% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane.

For control and treated samples, equal amounts of protein were loaded in the SDS-PAGE. After blocking, membranes were incubated overnight at 4°C with polyclonal antibodies for total and phosphorylated NFATC3 (1:10,000; Cell Signaling, Dublin, Ireland). Membranes were reproped with either anti-β-actin or GAPDH antibody (Cell Signaling) as loading controls. The blots were then processed according to standard protocols using ECL detection system (Fisher Scientific, Dublin, Ireland).

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA from LC cells was extracted with TRIzol (Life Technologies, Dublin, Ireland); total RNA was converted to cDNA using enhanced avian reverse transcriptase (eAMV), and 2 μg total RNA was used for first-strand synthesis with oligo-dT, deoxynucleotides (dNTPs), and primers. The following genes were analyzed using primers designed on q Primer Depot and manufactured by Sigma: TGFβ1, Col1A1, and periostin (Table). To quantify cDNA expression levels, quantitative RT-PCR was performed using a Rotorgene 3000 Real-Time PCR Thermocycler (Labotechnik, Wasserburg, Germany). The cDNA was assayed in triplicate using QuantiTect SYBR Green PCR Master Mix (Qiagen, Manchester, UK). Isolated cDNA was subjected to quantitative PCR using the formula 2³⁻ΔΔCT in which CT is the cycle threshold number.⁵⁸

**Immunofluorescence Microscopy**

To demonstrate that CsA prevents the oxidative stress-induced NFATC3 nuclear translocation, we used immunofluorescence microscopy. The primary human LC cells (passages 4–8) were seeded and grown on the Nunc Lab-Tek II Chamber Slide System (Thermo Scientific, Dublin, Ireland) in DMEM growth medium until confluence was reached. Control nontreated LC cells treated with H₂O₂ and LC cells pretreated with CsA were used. Cells were fixed in 100% ice-cold methanol for 5 minutes, permeabilized in 0.05% Triton X-100, blocked in 10% goat serum, and rinsed three times with PBS. Cells were incubated overnight at 4°C with 1:50 dilution of mouse monoclonal p-NFATc3 (sc-365785; Santa Cruz) followed by three washes for 5 minutes with PBS, and then incubated in the dark for 1 hour at room temperature with 1:250 dilution goat anti-mouse IgG-Fab-specific FITC conjugate (F2653 Sigma Aldrich Ireland) followed by three washes for 5 minutes. Hoechst 33342 (Thermo Scientific) was used as a nuclear counterstain. Images were captured using an Olympus BX51 fluorescent microscope and associated CellSens software (Olympus, Cardiff, UK).

**Confocal Microscopy**

LC cells were seeded on MatTek glass bottom Petri dishes (MatTek, Ashland, MA, USA) at 1500 cells/cm² and grown in DMEM overnight. Loading with fluorescent sensor Fluo-4 FM (2.5 μM) was performed for 30 minutes in Opti-MEM medium in the dark; then the medium was replaced with fresh DMEM and cells were incubated for 30 minutes prior to microscopy. Confocal fluorescence imaging was conducted on the Olympus FV1000 confocal laser scanning microscope with controlled...
CO₂, humidity, and temperature. The Fluo-4 probe was excited at 488 nm (10% of maximal laser power) with emission collected at 500 to 540 nm. Changes in cytosolic Ca²⁺ levels in cells upon treatment with H₂O₂ (100 μM) were compared to that in cells pretreated overnight with CsA (10 μM). In all experiments, differential interference contrast (DIC) and fluorescence images were collected kinetically with a ×60 oil immersion objective in 12 planes using 0.25-μm steps and 4-minute intervals. The resulting z-stacked images were analyzed using FV1000 Viewer software (Olympus), Excel (Microsoft Corporation, Redmond, WA, USA), and Adobe Photoshop and Illustrator (Adobe Systems, Inc., San Jose, CA, USA).

Statistical Analysis

Data were expressed as means of independent experiments, with SE error bars, or representative individual experiments. Statistical significance was analyzed by Student’s t-test (2-tailed; paired or unpaired as appropriate) for comparison between two groups and by 1-way analyses of variance (ANOVA) with Tukey-Kramer posttest for multiple comparisons. *P < 0.05 was taken as the level of significance (*P < 0.05, **P < 0.01). N refers to the number of eye donors, and n refers to the number of the cells in one experiment. Calculations were performed by the Origin 7.0 software (OriginLab, Stoke Mandeville, Bucks, UK). Densitometric analysis of Western blots was performed using Gene Tools software (Syngene, Cambridge, UK). For confocal microscopy experiments, differences in Fluor-4 intensities were analyzed using the Mann-Whitney U test.

RESULTS

Basal and Hypotonic Stretch [Ca²⁺] i Levels Are Elevated in Glaucoma LC Cells

Our recent work reported the effect of hypotonic stretch on [Ca²⁺] i in normal LC cells. Here we wished to compare the basal and hypotonic cell membrane stretch-induced changes in [Ca²⁺] i between normal and glaucoma LC cells. Using radiometric dual-wavelength imaging on Fura-2-AM–loaded LC cells, basal [Ca²⁺] i was first measured in isotonic solution for 5 minutes to obtain a stable baseline, and then hypotonic solution was applied to create hypotonic cell membrane stretch, as glaucoma-related stimulus, and [Ca²⁺] i measurement was recorded for 20 minutes. As shown in Figure 1, the basal [Ca²⁺] i was significantly greater in glaucoma LC cells (129.18 ± 8.22 nM) versus normal LC cells (108 ± 4.96 nM) (n = 9 independent experiments, 80 cells; *P < 0.05). On average, the hypotonic stimulus evoked an [Ca²⁺] i increase to 153.46 ± 21.29 nM in normal LC cells and to 339.5 ± 25.6 nM in glaucoma LC cells. The average peak amplitude in response to hypotonic stretch of cells from seven experiments obtained from seven normal and seven glaucoma eye donors was significantly greater in the glaucoma LC cells (P < 0.01).

Delayed Reuptake of Intracellular Ca²⁺ Stores in Glaucoma LC Cells

Having shown that Ca²⁺ entry results in a large increase in [Ca²⁺] i in glaucoma cells (Fig. 1), we next examined whether the extracellular Ca²⁺ is part of the abnormally elevated levels of [Ca²⁺] i, found in glaucoma LC cells.

As shown in Figure 2, we experimentally separated intracellular Ca²⁺ release from extracellular Ca²⁺ influx. Ca²⁺ influx was measured in cells loaded with Fura-2-AM. At time 0, the cells were bathed in Ca²⁺-free isotonic standard solution with 2 mM EGTA (to chelate the extracellular Ca²⁺) and 1 μM TG (to block SERCA pumps, inducing a complete depletion of Ca²⁺ from intracellular stores). As a result, [Ca²⁺] i transiently increased and was followed by a return to low baseline levels in both normal and glaucoma LC cells (Figs. 2A, 2B). However, in normal LC cells, the [Ca²⁺] i returned to basal level within 2 minutes, while the return to baseline took 12 to 14 minutes in glaucoma LC cells (Figs. 2A–C). When [Ca²⁺] i was stable again, the extracellular medium was replaced by hypotonic solution and extracellular CaCl₂ (2 mM) was added, resulting in an influx of Ca²⁺ via Ca²⁺ entry channels. As shown in Figure 2B, the rise of [Ca²⁺] i after changing the medium to hypotonic solution and reintroducing extracellular Ca²⁺ was
significantly greater in glaucoma LC cells ($P < 0.01$), and no recovery to baseline levels was observed over the time course studied in the glaucoma LC cells (Fig. 2C).

Expression Analysis of NFATc3 in Normal and Glaucoma LC Cells

Using an antibody against the total (phospho + nonphosphorylated) NFATc3, we found that NFATc3 protein expression was upregulated approximately 4-fold ($n = 3$, $P \leq 0.01$) in glaucoma when compared to that in normal LC cells (Fig. 3A) (although the normal LC donor N2 showed a moderate increase in NFATc3 expression level).

We next determined whether the expression of NFATc3 in normal and glaucoma LC cells was sensitive to an oxidative stress stimulus. LC cells obtained from different normal and glaucoma eye donors were pretreated with H$_2$O$_2$ (100 µM) for 6 hours, and the levels of NFATc3 protein expression in treated and nontreated LC cells were assessed using Western blotting analysis. Figures 3B and 3C show that NFAT levels were similarly increased in both normal and glaucoma LC cells following H$_2$O$_2$ pretreatment.

Cyclosporin A Reduces the H$_2$O$_2$-Induced Increase in NFATc3 Protein Expression, ECM Gene Transcription, and NFATc3 Dephosphorylation

A control experiment was first conducted to determine the effective dose of CsA on NFATc3 expression in H$_2$O$_2$-treated normal LC cells. Western blotting analysis using an antibody against the total (phospho + nonphosphorylated) NFATc3 demonstrated that 10 µM CsA pretreatment of normal LC cells inhibited the H$_2$O$_2$-induced increase in NFATc3 protein levels (Fig. 4), while low concentrations of CsA (1, 2, 5 µM) failed to block the H$_2$O$_2$-induced increase in NFATc3 protein levels. Based on these data, we used 10 µM CsA in all subsequent experiments.
NFAT Signaling Pathway in Glaucoma Lamina Cribrosa Cells

![Image of Western blot analysis](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/936739/)

**Figure 3.** Western blot analysis of NFATc3 expression in normal and glaucoma LC cells. (A) Representative example of Western blot analysis of total NFATc3 expression in normal LC cell strains (n = 3) and glaucoma cell strains (n = 5). Protein lysates were examined for differential expression of the total (phospho + unphosphorylated) NFATc3 protein. Expression of NFATc3 is significantly (**P < 0.01** increased in glaucoma (G1, G2, G3) compared to normal control (N1, N2, N3) LC cells. (B) Representative illustration of Western blot analysis of NFATc3 expression in normal and glaucoma LC cells exposed (or not) to H2O2 (100 µM) for 6 hours to induce a sustained oxidative stress. Note that protein lysates examined showed that the NFATc3 protein expression level is similarly increased by H2O2 pretreatment in both normal and glaucoma LC cells. (C) Summarized data illustrating the average protein levels of total NFATc3 in two different cell strains for control non-treated and H2O2 (100 µM)-treated normal and glaucoma LC cells (**P < 0.01, 1-way ANOVA with Tukey-Kramer posttest). Results are expressed as relative pixel intensity versus normal LC samples. GAPDH was used as a loading control. Values that are significantly different from control are denoted by an asterisk (**P < 0.05, **P < 0.01).

We next evaluated whether CsA reduced the profibrotic ECM gene expression. CsA pretreatment significantly reduced H2O2-induced expression of the profibrotic ECM genes tested (TGFβ1, Col1A1, and peristin) (n = 3, P < 0.01) (Fig. 5), both in normal and in glaucoma LC cells.

In a follow-up experiment, we evaluated whether CsA contributes to the profibrotic ECM gene expression by a mechanism involving the Ca2+-calcineurin/NFAT signaling pathway in normal and glaucoma LC cells. Western blotting experiments were performed in untreated controls and H2O2-treated normal and glaucoma LC cells in the presence or absence of CsA (10 µM), using an anti-phosphorylated NFATc3 antibody (pNFATc3). Western blot analysis shows that H2O2 significantly reduced the phosphorylated NFATc3 expression levels and this reduction was reversed by pretreatment with CsA (Fig. 6). To confirm these results, we used immunofluorescence microscopy to test whether H2O2 treatment induced cytoplasmic NFATc3 dephosphorylation and examine whether this dephosphorylation is inhibited by CsA pretreatment. Figure 7 illustrates a decrease in the cytoplasmic levels of phosphorylated NFATc3 when the LC cells were treated with H2O2, and this decrease was prevented by pretreating the LC cells with CsA, suggesting that NFATc3 nuclear translocation occurred.

As shown in Supplementary Figure S1, we also found a marked increase in global [Ca2+]i, levels following exposure to 100 µM H2O2 in normal LC cells. In CsA-treated normal LC cells, the H2O2-mediated elevation of intracellular Ca2+ was significantly decreased (n = 3, P < 0.05).

**Discussion**

In this study, we found elevated levels of basal and hypotonic cell membrane stretch-induced [Ca2+]i, with delayed reuptake in glaucoma LC cells, and this was associated with increased expression of NFATc3 in glaucoma LC cells. Both normal and glaucoma LC cells were sensitive to an oxidative stress stimulus. Pretreatment with CsA significantly reduced fibrosis gene (TGFβ1, Col1A1, and peristin) expression and reversed the H2O2-induced reduction in p-NFATc3 both in normal and in glaucoma LC cells.

Changes in cytosolic Ca2+ concentration play a vital role in intracellular signaling pathways related to proliferation, differentiation, apoptosis, and gene expression.40 In normal cells, the intracellular Ca2+ concentration is tightly regulated and kept relatively constant through a variety of homeostatic mechanisms.40 Ca2+ influx from the extracellular space and Ca2+ release from the intracellular sources determine the [Ca2+]i. We previously used single-wavelength flow cytometry (which is most useful for quantitative measurements of [Ca2+]i) in a population of cells to show that intracellular Ca2+ levels were higher in glaucoma LC cells compared to normal controls.12,30 In our current study, we used the radiometric dual-wavelength indicator Fura 2-AM (which has the advantage that it can be used for both quantitative and qualitative data) to measure changes in [Ca2+]i. Extracellular Ca2+ influx and/or release of Ca2+ from intracellular stores are the two sources of increased [Ca2+]i. We first examined whether this elevated cytosolic Ca2+ in glaucoma LC cells could have resulted from extracellular Ca2+ influx. We found that introduction of extracellular Ca2+ to the bathing solution increased [Ca2+]i, in
both basal and hypotonic cell stretch conditions, and this increase was more pronounced in glaucoma compared to normal LC cells (Fig. 1). The LC cell response to hypotonic stretch in the presence of extracellular Ca²⁺ was a typical response characterized by an initial Ca²⁺ transient spike phase, which is generated by inositol trisphosphate receptor (IP3-R)-dependent Ca²⁺ release from the intracellular stores. The resulting depletion of the Ca²⁺ stores by TG generates the activation of several types of cation channels such as store-operated channels, leading to the sustained [Ca²⁺]ᵢ increase by initiating extracellular Ca²⁺ influx.

Having demonstrated the contribution of extracellular Ca²⁺ to the abnormally raised [Ca²⁺]ᵢ in glaucoma LC cells, we next tested whether the release of Ca²⁺ from intracellular stores could also contribute to the abnormally elevated levels of [Ca²⁺]ᵢ, found in glaucoma LC cells. Therefore, we experimentally separated intracellular Ca²⁺ release from extracellular Ca²⁺ influx. The first part of the experiment (Fig. 2) showed that the release of Ca²⁺ from intracellular stores also contributed to the defective Ca²⁺ homeostasis found in glaucoma LC cells, as removal of the residual external Ca²⁺ from the external medium with EGTA followed by TG application resulted in a transient rise of [Ca²⁺]ᵢ, with similar amplitudes in glaucoma and normal LC cells. However, in normal LC cells, the [Ca²⁺]ᵢ returned to basal levels approximately 2 minutes after TG application, while return to baseline took 12 to 14 minutes in the glaucoma cells, indicating a lack of refilling of intracellular stores in glaucoma LC cells. These results complement our previous work, where an increase in SERCA2 and SERCA3 protein expression levels was observed in glaucoma LC cells. Interstingly, the second part of the experiment (Fig. 2) showed that the addition of extracellular Ca²⁺ to the hypotonic buffer markedly increased the Ca²⁺ amplitude of the sustained phase of Ca²⁺ elevation more in the glaucoma than in normal LC cells. This suggests that the Ca²⁺ entry channels are operating in a high open probability gating mode, thus generating persistent Ca²⁺ influx in glaucoma LC cells. Furthermore, and more importantly, the normal LC cells recovered to baseline Ca²⁺ levels in contrast to the glaucoma LC cells after changing back to isotonic solution, suggesting a defect of Ca²⁺ extrusion from the intracellular space. The key components that maintain stable Ca²⁺ gradients in cells are believed to be plasma membrane Ca²⁺ entry channels, ATP-driven pumps (PMCAs), electrogenic exchangers Na⁺-Ca²⁺ (NCX), and SERCA pumps. In our previous study, we also found that the Ca²⁺ extrusion system is altered in glaucoma LC cells. Our data showed that NCX expression levels were significantly lower in glaucoma LC cells, suggesting a defect in Ca²⁺ extrusion from the intracellular space.

**Figure 4.** Effect of cyclosporin A on H₂O₂-induced NFATc3 expression in normal LC cells. LC cells were preincubated overnight with different concentrations of CsA (0, 1, 2, 5, 10, 100 μM) and cultured at 37°C with or without H₂O₂ (100 μM) for 6 hours to induce a sustained oxidative stress. (A) Representative Western immunoblotting performed using an anti-NFATc3-specific antibody. (B) Summarized data of total NFATc3 expression in three different LC cell strains. Note that CsA 10 μM completely blocked NFATc3 expression. Results are expressed as relative pixel intensity versus control LC samples. GAPDH was used as a loading control. Values significantly different from control are denoted by an asterisk (*P < 0.05, **P < 0.01).
Our results showing elevated [Ca\(^{2+}\)]\(_i\) levels in glaucoma LC cells are very similar to findings in TM cells.\(^{37}\) There is strong evidence of increased ECM production in the glaucomatous TM\(^{41}\) resulting in a reduced outflow facility. The paper by He and colleagues\(^{37}\) shows an increase in cytosolic and mitochondrial Ca\(^{2+}\) in primary open-angle glaucoma (POAG) TM cells with evidence of mitochondrial dysfunction. The link between the anterior and posterior segments in glaucoma was well discussed in a recent review paper by Sacca and colleagues.\(^{42}\) Of interest also are papers by Sappington et al.\(^{43}\) and Niittykoski et al.,\(^{44}\) who also demonstrated increased [Ca\(^{2+}\)]\(_i\) levels in glaucomatous retinal ganglion cells (RGC).\(^{45,44}\) Other work in a rodent ocular hypertension model showed that calcineurin is activated in RGCs and participates in RGC death,\(^{45}\) which was prevented by CsA treatment.\(^{45}\)
Cellular responses to Ca\textsuperscript{2+} mobilization are highly versatile due to the capacity of intracellular Ca\textsuperscript{2+} signaling to activate an extensive repertoire of downstream signaling targets, including NFAT and other pathways such as PKCa and MAPK. Consequently, any long-term increase in [Ca\textsuperscript{2+}]\textsubscript{i} will have larger knock-on effects in the intracellular signaling pathways, and we believe that the small but statistically significant increase in baseline [Ca\textsuperscript{2+}]\textsubscript{i} will have a larger impact on, for example, the NFAT pathway as shown in Figure 3.

In addition, intracellular Ca\textsuperscript{2+} levels play a key role in neurodegenerative diseases. Several lines of evidence indicate that intracellular Ca\textsuperscript{2+} levels are elevated in Alzheimer's disease.\textsuperscript{46} A rise in [Ca\textsuperscript{2+}]\textsubscript{i} levels results in subsequent defects in neuronal Ca\textsuperscript{2+} signaling. Altered Ca\textsuperscript{2+} signals shift the balance between Ca\textsuperscript{2+}-dependent phosphatase calcineurin and...
Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), which are extremely abundant in synaptic locations. A change in the balance of CaMKII and calcineurin activity causes synaptic and memory damage that subsequently lead to synaptic loss and neurodegeneration. Despite the differences in mechanisms of cell loss, most of the neurodegenerative disorders share some common characteristics, including the contribution of mitochondrial dysfunction and oxidative stress in the development of pathology and, specific for each disease, misfolded proteins that aggregate in the brain.47 Being the main energy producer in the cell, mitochondria play an important role in the mechanism of apoptosis, Ca\(^{2+}\), and redox signaling.48 The ability of mitochondria to produce reactive oxygen species (ROS) in the electron transport chain has a functional consequence in cell signaling; nevertheless, overproduction of ROS in mitochondria links this organelle to age-related pathology and neurodegenerative disorders.49 In addition, mitochondria also provide a direct link between environment and genes.50

We consequently examined and compared the NFATc3 isoform protein expression between normal controls and glaucoma LC cells. NFATc3 protein expression is upregulated approximately 4-fold (\(P < 0.01\)) in glaucoma LC cells. In addition, both normal and glaucoma LC cells were sensitive to an oxidative stress (H\(_2\)O\(_2\)) stimulus, as the NFATc3 protein expression levels were similarly increased in both normal and glaucoma LC cells following H\(_2\)O\(_2\) treatment. NFAT is a Ca\(^{2+}\)-dependent transcription factor that regulates the expression of specific genes through a calcineurin-dependent signaling pathway in various cell types.19 The NFAT family of transcription factors are highly phosphorylated in the cytoplasm of resting cells. However, an increase of [Ca\(^{2+}\)]

\[\text{Cell Membrane} \]
\[\text{Oxidative Stress} \]
\[\text{TGFβ} \]
\[\text{Mechanical Stress} \]
\[\text{Ca}^{2+} \]
\[\text{Calcineurin} \]
\[\text{Cyclosporin A} \]
\[\text{Nucleus} \]
\[\text{ECM Gene Expression} \]
\[\text{Excessive ECM Gene production} \]
\[\text{Pathological Wound Healing} \]
\[\text{Fibrosis} \]
\[\text{Regulated ECM Gene production} \]
\[\text{Physiological Wound Healing} \]
\[\text{Resolution of Inflammation} \]

**Figure 8.** Proposed mechanism of profibrotic gene transcription regulation via the calcineurin/nuclear factor of the activated T cells (NFATc3) signaling pathway in LC cells. Intracellular Ca\(^{2+}\) levels increase in response to stimuli such as TGFβ, oxidative stress, and cell membrane stretch resulting in calmodulin saturation, which in turn promotes calcineurin activation. Activated calcineurin dephosphorylates several substrates, including the NFAT transcription factors, which then translocate from the cytoplasm to the nucleus and activate Ca\(^{2+}\)-inducible genes including those contributing to fibrosis in glaucoma. CsA inhibits calcineurin activation, thereby preventing NFAT dephosphorylation and inhibiting fibrosis. In LC cells, this include ECM genes, the protein products of which inhibit calcineurin, resulting in a negative feedback loop.
Given the extensive number of studies demonstrating a key role for NFAT in driving hypertrophic gene expression in cardiac tissues and fibrosis,17–22 we speculate that NFAT plays a similar role in LC cells in glaucoma. In addition to its role in the immune system and cardiac tissues, NFATc3 plays a key role in activating the Ca\(^{2+}\)/calmodulin/calcineurin/NFAT signaling pathway in pulmonary arterial smooth muscle cells in models of pulmonary hypertension.51–53

In the present study, our findings revealed that the blockade of NFATc3 activation with CsA resulted in the downregulation of H\(_2\)O\(_2\)-induced profibrotic gene expression including TGF\(\beta\)1, Col1A1, and periostin in normal and glaucoma LC cells. We also demonstrated that CsA pretreatment significantly reversed the H\(_2\)O\(_2\) reduction in phosphorylated NFAT and its nuclear translocation, indicating that CsA reduces the H\(_2\)O\(_2\)-induced profibrotic gene expression through a mechanism involving the Ca\(^{2+}\)/calcineurin/NFAT signaling pathway in normal and glaucoma LC cells.

In addition, we further examined the effect of CsA on H\(_2\)O\(_2\)-induced elevation on “global” [Ca\(^{2+}\)]\(_i\) levels using confocal imaging where the LC cells were loaded with Fluo-4 to detect cytoplasmic Ca\(^{2+}\) changes (Supplementary Fig. S1). Treatment of LC cells with CsA resulted in a significant reduction of the global cytosolic Ca\(^{2+}\) induced by oxidative stress in normal LC cells.

In the present study, we utilized two glaucoma-related stimuli (oxidative stress and hypo-osmotic cell membrane stretch) to assess calcium homeostasis in LC cells. Previous glaucoma investigations have identified a key role for oxidative stress37,54 and cell stretch.39,55 Oxidative and osmotic stress stimuli (oxidative stress and hypo-osmotic cell membrane stretch) to assess calcium homeostasis in LC cells. Previous studies have shown that oxidative stress and cell membrane stretch can activate growth factor expression59,60 and profibrotic ECM proteins.61,62

In conclusion, we found elevated NFAT expression in glaucoma LC cells. CsA inhibited the oxidative stress–induced increase in NFAT expression and transcription activity and also ECM profibrotic gene expression in normal and glaucoma LC cells. This suggests that inhibition of the NFAT signaling pathway may reduce LC fibrosis in glaucoma (Fig. 8).

Acknowledgments

Supported by the 2014 Shaffer Grants for Innovative Glaucoma Research Foundation.

Disclosure: M. Irnaten, None; A. Zhdanov, None; D. Brennan, None; T. Crotty, None; A. Clark, None; D. Papkovsky, None; C. O’Brien, None

References
