Mitochondrial Defects and Dysfunction in Calcium Regulation in Glaucomatous Trabecular Meshwork Cells

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PURPOSE. Disruption in intracellular calcium ion (Ca$^{2+}$) homeostasis has major effects on health. Persistent Ca$^{2+}$ overload induces mitochondrial permeability transition pore (MPTP) opening, which prompts mitochondrial release of calcium (mCICR) and reactive oxygen species (ROS) into the cytosol which, in turn, compromises mitochondrial function. This study was conducted to examine intracellular Ca$^{2+}$ levels and mitochondrial vulnerability to Ca$^{2+}$ stress in trabecular meshwork (TM) of individuals with primary open-angle glaucoma (POAG).

METHODS. Primary cultures of TM cells from POAG (GTM) and age-matched, nondiseased (NTM) eyes, obtained from post-mortem donors eyes by standard surgical trabeculectomy, were treated with the following calcium regulators: the mitochondrial respiratory chain 1 inhibitor rotenone (ROT); the mitochondrial permeability transition pore (MPTP) inhibitors cyclosporine (Cys) and aristolochic acid (ArA); the Ca$^{2+}$ chelators BAPTA/AM or EDTA; the mitochondrial Ca$^{2+}$ uniporter inhibitor ruthenium red (RR); the Ca$^{2+}$/Na$^{+}$ exchanger inhibitor trifluoperazine; and the inositol 1,4,5-triphosphate receptor type 3 (IP3R) inhibitors 2-aminoethoxydiphenyl borane (2-APB) and xestospongin C (Xe-C). Ca$^{2+}$ concentrations in the cytoplasm ([Ca$^{2+}$]_c) and mitochondria ([Ca$^{2+}$]_m) were determined by confocal microscopy and flow cytometry with the fluorescent Ca$^{2+}$ indicators fluo-3/AM and rhod-2/AM, respectively. Mitochondrial membrane potential (ΔΨm) was examined with the fluorescent probe tetramethylrhodamine ethyl ester (TMRE). The expression of cyclophilin D, a protein that induces MPTP opening was also measured.

RESULTS. There was increased [Ca$^{2+}$]_c, [Ca$^{2+}$]_m, mCICR, MPTP opening, and expression of cyclophilin D and decreased ΔΨm in POAG TM cells compared with control cells. ROT artificially exacerbated these conditions in GTM cells. Chelation of [Ca$^{2+}$]_c and inhibition of IP3R and MPTP opening suppressed mitochondrial dysfunction and reduced the additional effects of ROT in GTM cells.

CONCLUSIONS. POAG TM cells have defective mitochondrial function, which causes them to be abnormally vulnerable to Ca$^{2+}$ stress. The dysfunction in calcium regulation by these cells may contribute to the failure of this tissue to control IOP. Pharmacologic inhibitors of IP3R, MPTP opening, and cyclophilin D could have clinical implications for primary open-angle glaucoma. (Invest Ophthalmol Vis Sci. 2008;49:4912–4922 DOI:10.1167/iovs.08-2192)

Glaucoma is a chronic ocular disease frequently associated with increased intraocular pressure (IOP) caused by the anatomic restriction of fluid drainage through the major outflow pathway of the eye.1,2 The disease affects approximately 67 million people worldwide and is the second leading cause of irreversible blindness.3

The tissue that regulates the flow of aqueous humor and IOP is the trabecular meshwork (TM), a soft spongiform structure found in the iridocorneal angle of the eye. TM cells are highly contractile and use tightly controlled mechanisms associated with phagocytosis, cytoskeletal reorganization, cell adhesion, matrix deposition, and ion channel transport to maintain physiological aqueous humor resistance.4–6 Disruption in the function of these cells results in a failure to control IOP levels, a hallmark in the development of glaucoma.4,5,7

There are suggestions that calcification in the TM underlies the pathologic progression of glaucoma. In support of this notion, there is evidence of increased activity of the vascular calcification marker alkaline phosphatase in the TM of individuals with glaucoma8 and decreased expression of an inhibitor of calcification, matrix Gla protein (MGP), a protein that plays a key role in the formation of atherosclerotic plaques and vascular calcification.9,10 TM cells also express the atherosclerosis markers, cartilage GPO-39 and endothelial leukocyte adhesion molecule (ELAM)-1.11 High expression of calcification markers in the TM of patients with glaucoma suggests that there is a correlation between the process of calcification and the development of glaucoma.8

Alterations in calcium homeostasis and dysfunction in calcium buffering systems have implications for the health and function of many types of cells.12,13 Calcium ions (Ca$^{2+}$) represent a second messenger that integrates many important signaling pathways, including those that affect cell death and proliferation, neurotransmitter release, and cellular excitability.14–16 The mitochondria can transiently accumulate high concentrations of Ca$^{2+}$ by uptake through the potential-driven calcium uniporter.17,18 In pathologic conditions, excessive entry of calcium into the mitochondria triggers opening of the mitochondria permeability transition pore (MPTP)19,20 and mitochondrial Ca$^{2+}$-induced calcium release (mCICR), which, in turn, can trigger further MPTP opening.21–23 MPTP opening requires high levels of intracellular Ca$^{2+}$ and an association of several proteins that form the pore, including cyclophilin D.24 Stress on the endoplasmic reticulum is another factor shown to induce opening of the MPTP.25

Induction of the MPTP opening results in mitochondrial swelling, increase in the generation of reactive oxygen species

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that the TM cells of eyes with POAG contain higher cytoplasmic calcium (Ca^{2+}) compared to non-POAG eyes. The mechanism of the increased Ca^{2+} in POAG TM cells is largely dependent on a defect in mitochondrial calcium dysregulation.

In this study, we examined whether there is a dysregulation in intracellular calcium levels in the TM cells of patients with primary open-angle glaucoma (POAG), as an extension of our recent observations that there is a defect in mitochondrial complex I that leads to increased ROS production and decreased ATP levels in the TM cells in glaucoma. Moreover, we report that the TM cells of eyes with POAG contain higher cytoplasmic and mitochondrial levels of Ca^{2+} when compared with that of non-POAG eyes. The mechanism of this dysregulation may protect the TM and restore normal IOP in patients with glaucoma.

**Materials and Methods**

All tissue culture reagents were obtained from Invitrogen-Gibco (Gaithersburg, MD). Dexamethasone (DEX), rotenone (ROT), cyclosporine (Cys), aristolochic acid (ArA), EDTA disodium salt solution (EDTA), 1,2-Bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA/AM), ruthenium red (RR), trifluoperazine, 2-aminoethoxydiphenyl borane (2-APB), and xestospongin C (Xe-C) were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-3/AM, rhod-2/AM, and tetramethylrhodamine ethyl ester (TMRE) were obtained from Invitrogen-Molecular Probes (Eugene, OR). Myocilin gene was designed with commercial software (OligoPerfect Designer Software; Invitrogen, Carlsbad, CA) and synthesized by Invitrogen. The identity of the primary TM cells in the cultures was examined according to published procedures, by using well-characterized polyclonal or monoclonal antibodies to the proteins FN, LN, and NSE, and by the ability of TM cells to upregulate myocilin expression after DEX treatment.48,49 For inducible myocilin expression in TM cells, the cultures were treated with DEX (10^{-7} M) for 5 days.

**Patient Information**

Before surgery, clinical data were collected for each patient, including age, sex, use of prostaglandin analogues, number of argon laser trabeculoplasties and other ocular surgical interventions, type and duration of glaucoma, IOP, and visual acuity. Glaucoma diagnosis was based on the clinical examination, including slit lamp, optical coherence tomography, gonioscopy, fundus photography, and visual field perimetry. All patients underwent slit lamp examination again the day before surgery. All IOPs in the POAG group exceeded 20 mm Hg at the time of surgery. Visual acuity varied from 0.3 to 1.0. There is a full ophthalmic history of each donor eye available at the Eye Bank of Zhongshan Ophthalmic Center (http://www.zscophab.com/I1.html).

According to the records, no known eye diseases had been detected in the control samples used in the present study. The TM tissues for the POAG samples were obtained from individuals with a similar stage of glaucoma after diagnosis by glaucoma specialists. These patients received the prostaglandin analogues latanoprost (0.005%) and travoprost (0.004%) for similar lengths of time. Prostaglandins have been reported to increase trabecular outflow. None of the individuals from whom the TM samples were obtained had received steroid medications. The average duration of glaucoma in the patients with POAG was approximately 2 years, and none had a record of systemic disease. Tissue from each patient was used to generate primary cultures of TM cells, as described.

**Tissue Procurement and Cell Culture**

All human tissue used in this study was procured and managed in accordance with the tenets of the Declaration of Helsinki and with approval from the Institutional Review Board of the Sun Yat-sen University at Guangzhou. Normal human eyes were obtained from the Zongshan Ophthalmic Center Eye Bank in Guangzhou, China. All patients enrolled in the study provided informed written voluntary consent.

Normal TM cells were derived from eight human donor eyes, without any known ocular diseases. The eyes were used for corneal transplantation and harvested less than 24 hours after death from donors between 20 to 60 years of age. TM specimens from eight patients with POAG (age range, 15–60 years) were obtained by surgical trabeculectomy according to standard surgical procedures as described elsewhere. Briefly, a large flap was used (scleral flap size: 8–10 × 5–8 mm, flap thickness: two thirds scleral thickness; excised inner block size: 5 × 5 × 8 mm) allowing us to obtain a large sample size. We used a surgical meridian section to obtain the TM that was trapeziform in shape, with one side at Schwalbe’s line and the other at its base at the sclera spur and/or cornea-sclera limbus. The meshwork consists of a stack of flattened, interconnected, perforated sheets, which run in meridian fashion from the peripheral cornea and Descemet’s membrane anterior to the scleral spur.41 To remove all specimens, we used a 45° knife to cut a 5 × 5 × 8-mm button of corneoscleral tissue. The TM was then dissected under microscopic control.

The TM tissue of each of the eight nondiseased control eyes and eight POAG eyes was used to generate independent primary cultures of TM cells. The samples were never pooled in these experiments. The explants were immediately placed in 24-well culture plates (Corning Costar, Cambridge, MA) containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) as previously described.42 Cells from the TM migrated from the explants and formed a confluent monolayer between 2 and 5 days. Cells from the explants were subsequently trypsinized, centrifuged, resuspended in complete DMEM, and seeded at a density of 1 × 10^5 cells/well in six-well tissue culture plates. Primary cultures from each of the eight POAG and eight control eyes were used at passages 3 to 4. Each treatment was repeated six times for each donor sample (n = 48). Micrographs of the cultures were taken 3 days after seeding, at approximately 80% confluence.

The identity of the primary TM cells in the cultures was examined according to published procedures, by using well-characterized polyclonal or monoclonal antibodies to the proteins FN, LN, and NSE, and by the ability of TM cells to upregulate myocilin expression after DEX treatment. For inducible myocilin expression in TM cells, the cultures were treated with DEX (10^{-7} M) for 5 days.

**Cellular Ca^{2+} Levels and ΔΨm**

Cellular Ca^{2+} levels and ΔΨm were measured in nontreated cultures and in those treated with the various agents listed earlier, after 30, 60, 90, and 120 minutes, respectively.

To determine the source of cytosolic free calcium ion (Ca^{2+}) and the expression of cyclophilin D in nondiseased and POAG TM cells, we used the first 24- and the second 24-h cultures and in those treated with the various agents listed earlier, after 30, 60, 90, and 120 minutes, respectively.

The NTM50 and GTRM50-52 cell lines. These transformed human TM cell lines were a generous gift from Prof. Jou-Hou Pang (Glaucoma Research, Alcon Research, Ltd, Fort Worth, TX). We used these cell lines because they are readily available and because they have features characteristic of the respective primary TM cell cultures. The TM cell lines were cultured identically with the primary human TM cells.
Characterization of Cultured TM Cells

Immunolabeling studies were performed with specific markers used as a molecular index to confirm that the primary cultures obtained from normal and POAG eyes and the cell lines were indeed TM cells. The expression of FN, LN, and NSE was analyzed in the primary cultures. Briefly, TM cells were seeded onto polylysine (10 μg/mL)-coated glass chamber slides at a density of 2000 cells/chamber. After the cultures were rinsed with PBS, the cells were fixed in ice cold 4% paraformaldehyde for 15 minutes, then treated for 4 minutes in 100 mM phosphate buffer, 1 mg/mL bovine serum albumin (BSA), and 0.2% Triton X-100, to permeabilize the cell membranes. The endogenous peroxidase activity in the cells was then quenched with 3% H2O2 before the samples were incubated with 0.5% blocking reagent for 30 minutes ( TSA-Direct kit; DuPont-NEN, Boston, MA). After this procedure, the cells were immunolabeled with one of the following antibodies at room temperature for 1 hour: mouse monoclonal anti-FN (1:200), rabbit polyclonal anti-LN (1:200), and mouse monoclonal anti-NSE (1:200). Labeling was followed by several washes with PBS to remove the primary antibody, then incubation for an additional 45 minutes with biotinylated goat anti-mouse IgG (1:500, Vector Laboratories, Burlingame, CA) or anti-rabbit IgG (1:500, Vector Laboratories) where appropriate, before reaction with the avidin-biotin-peroxidase complex for 10 minutes. We also incubated the TM cells with the secondary antibody alone as the negative control. After a series of washes, the specimens were treated with 3,3′-diaminobenzidine (DAB)/peroxidase reaction (Vector DAB substrate kit; Vector Laboratories), washed, treated with hematoxylin counterstain, washed again, and then dried at room temperature. The samples were then dehydrated in a graded series of alcohols and coverslipped with 1,3-diethyl-8-phenylxanthine (DPX). The staining pattern for each antibody was visualized by phase-contrast microscope (DM IRB; Leica, Wetzlar, Germany).

Myocilin Gene Expression

Myocilin mRNA expression was analyzed by polymerase chain reaction (RT-PCR). Total mRNA from NTM and GTM samples were isolated (RNAeasy reagents; Qiagen, Valencia, CA) according to the manufacturer’s protocol. First-strand cDNA was synthesized (Script cDNA synthesis kit; Bio-Rad). RT-PCR was performed (iTaq polymerase; Bio-Rad) at an annealing temperature of 59°C for 30 cycles for both the myocilin and the GAPDH primers. The primer sequences used in PCR reactions for the myocilin gene were (sense) 5′-CTGGAAAC-GCAAACCCAGAGA-3′ and (antisense) 5′-AATCCTGGAAAGCAGAATTCT-3′. GAPDH was used as the internal RNA loading control, and samples to which no reverse transcriptase (RTs) was added to the PCR experiments were used as the negative control, to confirm that amplification was RNA-dependent. PCR products were resolved by 1% agarose gel electrophoresis.

Measurement of [Ca2+]i and [Ca2+]m in TM Cells

Changes in [Ca2+]i were measured with the fluorescent probe fluo-3/AM, and Rhod-2/AM (Kd = 570 nM) was used to measure [Ca2+]m, as described previously. Fluorescence intensity after labeling was measured by using both flow cytometry and confocal microscopy. For flow cytometric analyses, the cells were cultured in six-well plates at a density of 1 × 10^5 cells/well and were loaded with either 1 μM fluo-3/AM for 30 minutes or 1 μM rhod-2/AM for 1 hour. The cells were then trypsinized, washed twice with cold PBS, resuspended in 200 μL PBS, and immediately analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm for fluo-3/AM and at an excitation wavelength of 549 nm and emission wavelength of 581 nm for Rhod-2/AM. The fluorescence intensity of 10,000 labeled cells was routinely collected for each analysis, and the data are expressed as the median fluorescence intensity in arbitrary units from the average of at least three separate experiments.

For confocal microscopic analyses, the cultures were grown in 35-mm-diameter Petri dishes at a density of 1 × 10^5 cells/dish. Treated and nontreated TM cells were loaded with 8 μM fluo-3/AM for 30 minutes and then washed with DMEM to remove excessive fluo-3/AM before imaging. Micrographs were captured by confocal microscopy (100M; Carl Zeiss Meditec, GmbH, Jena, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Measurement of ΔΨm

ΔΨm was measured by using TMRE, as described previously. TM cultures of 1 × 10^5 cells/well of a six-well plate were harvested after the various treatments and the cells incubated with 250 nM TMRE (diluted in serum-free DMEM) for 30 minutes at 37°C in the dark. All samples were rinsed twice in PBS and analyzed immediately by flow cytometry at an excitation wavelength of 568 nm and an emission wavelength of >590 nm for TMRE. Results are expressed in arbitrary units as the median fluorescence intensity from the average of at least three separate experiments.

Analysis of Cyclophilin D Expression

Cyclophilin D mRNA expression was analyzed by polymerase chain reaction (RT-PCR) and quantitated by real-time PCR. Total mRNA from NTM5 and GTM3 samples were isolated (RNAeasy reagents; Qiagen, Valencia, CA) according to the manufacturer’s protocol. First-strand cDNA was synthesized (Script cDNA synthesis kit; Bio-Rad). RT-PCR was performed (iTaq polymerase; Bio-Rad) at an annealing temperature of 58°C for 35 cycles for both cyclophilin D and GAPDH primers. The primers’ sequences for cyclophilin D were (sense) 5′-GACTGGGAAAA-CCTCCCTCAT-3′ and (antisense) 5′-ATGTTGGAATCTTACATTTCC-3′. GAPDH was used as the internal RNA loading control, and samples to which no reverse transcriptase (RTs) was added to the PCR experiments were used as the negative control to confirm that amplification was RNA-dependent. PCR products were resolved by 1% agarose gel electrophoresis. For Quantitative real-time PCR, the two-step amplifying protocol was used with SYBR green supermix solution (iQ Supermix; Bio-Rad). Both melting curve and gel electrophoretic analyses were used to determine amplicon homogeneity and quality of the data.

For Western blot analysis, NTM5 and GTM3 cells were lysed in lysis buffer (Cytobuster; Novagen, Madison, WI), and protein concentrations in the supernatant were estimated (DC Protein Assay Kit; Bio-Rad). Protein (30 μg) was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% (wt/vol) dried milk, the membranes were incubated with primary antibodies (1:1000) for 3 hours at room temperature (RT), followed by washing and incubation with HRP-conjugated secondary antibodies for 1 hour at RT. Bound antibody was determined by chemiluminescence (ECL system; Bio-Rad).

Statistical Analysis

All assays were performed in at least six separate experiments in triplicate and data are expressed as the mean ± SE. One-way ANOVA was performed, and statistical significance was set at P < 0.05.

RESULTS

In a prior study, we reported several pathologic features in cells of the TM obtained from individuals with POAG (GTM) when compared with age-matched healthy control subjects (NTM). Many of these features are associated with abnormal function of the mitochondria in the GTM cells. In that study, we showed that a defect in mitochondrial complex I in the GTM cells contributes to increased ROS generation, lower mitochondrial membrane potential (ΔΨm), and decreased ATP production. Based on those findings, we proposed that mitochondrial dysfunction and oxidative stress play a strong role in the development of POAG.

In the present study, we extended those investigations to determine calcium regulation by using ROT to further exacerbate disease in GTM cells. Our studies showed that the mitochondria-SER feedback loop that regulates intracellular calcium
levels is perturbed in GTM cells and involves chronic MPTP opening and IP3R dependent release of calcium.

Characterization of GTM and NTM cells in Primary Culture

TM cells were identified in primary cultures obtained from normal and POAG eyes, based on their expression of FN, LN, and NSE, characteristic features of these cells. No staining was found in control specimens where the primary antibody was omitted and the secondary antibody was used. Consistent with previous findings, myocilin gene expression in both TM cells from eyes with POAG and nondiseased eyes was induced by 10^{-7} M DEX after treatment for 3 days, as shown by RT-PCR.

Cultured GTM and NTM cells grew well in culture, although the GTM cells grew slower than the NTM cells. There were subtle morphologic differences between the diseased and nondiseased cells, as well. The difference is best shown in the confluent images of the phase-contrast micrographs shown in Figure 2A, in which the GTM cells appear much larger, more irregularly shaped, and more fibroblastic in appearance than do the nondiseased age-matched NTM cells.

\[ [\text{Ca}^{2+}]_c, [\text{Ca}^{2+}]_m, \text{and } \Delta \Psi_m \text{ in GTM Cells} \]

High ROS levels can trigger MPTP opening, resulting in increased intracellular calcium levels. Since we had observed an increase in ROS production in GTM cells, we determined the resting level of \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_m\) in POAG and nondiseased TM cell populations. \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_m\) were detected by using the fluorescence \text{Ca}^{2+} indicators fluo-3/AM and Rhod-2/AM, respectively, by both confocal microscopy and flow cytometry. The confocal images in Figure 2A, taken after labeling with fluo-3/AM, indicated that there was an overall higher level of calcium in GTM cells than in their nondiseased counterparts. The analysis by flow cytometry in Figure 2B confirms this finding and shows that the endogenous resting levels of \([\text{Ca}^{2+}]_c\) were 2.0-fold \((\pm 0.26) \ (P < 0.05)\) higher and that those of \([\text{Ca}^{2+}]_m\) (Fig. 2C), determined by Rhod-2/AM labeling, were 2.2-fold \((\pm 0.23) \ (P < 0.05)\) greater in GTM cells than in NTM cells.

Flow cytometric distribution of the fluorescence intensity of the \(\Delta \Psi_m\) indicator TMRE in the TM cultures supports our previous findings that the \(\Delta \Psi_m\) in GTM cells was lower by 6.29-fold \((\pm 0.90) \ (P < 0.05)\) in GTM than in NTM cells (Fig. 2D). All flow cytometry data are expressed as multiples of change in \([\text{Ca}^{2+}]_c\), \([\text{Ca}^{2+}]_m\), and \(\Delta \Psi_m\) levels of GTM compared with NTM and the results are expressed as the mean \(\pm SE\) of six different experiments for each donor \((n = 48)\).

The studies indicate that there was calcium dysregulation in the TM cells obtained from POAG eyes that was not seen in the normal eyes and suggest that this abnormality is a key mechanism underlying dysfunction of TM in glaucoma.

Effect of ROT on Intracellular Calcium Levels and \(\Delta \Psi_m\) in GTM Cells

We next treated both TM cultures with the mitochondrial complex I inhibitor ROT to examine the sensitivity of the cells.
to ROT and the effects of this inhibitor in boosting intracellular calcium levels in the diseased and nondiseased TM cells. ROT is a well-known inhibitor of mitochondrial complex I, is widely used to decrease ΔΨm, and increases oxidative stress in cells. In Figure 3A, we show that treatment of the cultures with 5 μM ROT for 1 hour had no effect on [Ca\textsuperscript{2+}]\textsubscript{c} in NTM cells but had an additive effect on the high existing [Ca\textsuperscript{2+}]\textsubscript{m} in GTM cells.

The data were further confirmed by flow cytometry, after Fluo-3/AM loading of the cultures, which showed that the levels of [Ca\textsuperscript{2+}]\textsubscript{c} in GTM cells were increased by 2.87-fold (±0.67) (P < 0.05) above the resting levels (Fig. 3A). NTM cells were less sensitive to ROT, and only after treatment with high concentrations of ROT (>5 μM) did we observe any elevation in [Ca\textsuperscript{2+}]\textsubscript{c} in NTM cells (data not shown). Similarly, treatment with ROT for 30 minutes had an additive effect on [Ca\textsuperscript{2+}]\textsubscript{m} in GTM cells (2.77-fold [±0.7] increase) (P < 0.05) over the resting levels, as determined by Rhod-2/AM loading and flow cytometric analysis (Fig. 3B).

The relative amounts of total TMRE fluorescence in the two TM populations after ROT treatment were also evaluated after ROT treatment for 1 hour (Fig. 3C). In this study, we observed an additional decline in ΔΨm by 8.19 fold (±1.93) (P < 0.05) in the treated versus the nontreated GTM cells. The data are expressed as multiples of change in [Ca\textsuperscript{2+}]\textsubscript{c}, [Ca\textsuperscript{2+}]\textsubscript{m}, and ΔΨm levels of GTM to NTM and as the mean ± SE of six different experiments for each donor (n = 48).

This study indicates that ROT had an additive effect on the resting levels of [Ca\textsuperscript{2+}]\textsubscript{c}, [Ca\textsuperscript{2+}]\textsubscript{m}, and ΔΨm in GTM cells and could be used to enhance the GTM condition to determine the mechanisms that induce calcium dysregulation in the GTM cells.

**Effect of Intracellular Ca\textsuperscript{2+} Chelation on ΔΨm**

We next determined whether the increase in [Ca\textsuperscript{2+}]\textsubscript{c} in GTM cells is due to extracellular calcium sources or to ROT-induced release of intracellular calcium stores and how this increased [Ca\textsuperscript{2+}]\textsubscript{c} affects ΔΨm. First we measured the ΔΨm after treatment of the cultures with the Ca\textsuperscript{2+} chelators BAPTA/AM (50 μM) or EDTA (0.5 mM), alone or in the presence of 5 μM ROT. BAPTA-AM, a cell-permeable derivative of BAPTA, is widely used as an intracellular calcium sponge\textsuperscript{57-58} and mainly chelates the cytoplasmic pool of labile Ca\textsuperscript{2+} whereas EDTA mainly soaks up the extracellular pool of Ca\textsuperscript{2+}.

The results from these experiments are shown in Figure 4 and indicate that chelation of intracellular Ca\textsuperscript{2+} by BAPTA/AM prevented the decline of ΔΨm in untreated GTM cells and in those treated with ROT by 1.78- and 2.13-fold, respectively (Fig. 4A), whereas removal of the extracellular Ca\textsuperscript{2+} by EDTA had little effect on ROT-induced elevation of [Ca\textsuperscript{2+}]\textsubscript{c} levels (Fig. 5) and ΔΨm in GTM cells (Fig. 4B).

Our data imply that release of excess calcium from intracellular calcium stores contributes to the decrease in ΔΨm that we observed in the GTM cells and may exacerbate the disease in this tissue in vivo. The reduced ability of ROT to further decrease the ΔΨm in the absence of intracellular calcium supports this hypothesis.

**SER IP3R Mediation of the Increase in [Ca\textsuperscript{2+}]\textsubscript{c} in GTM**

From the studies cited herein, we know that ROT (5 μM) increases [Ca\textsuperscript{2+}]\textsubscript{c} in GTM but not in NTM cells and that extracellular chelation of calcium does not change this effect. Since the endoplasmic reticulum is a major storage site for intracellular calcium and Ca\textsuperscript{2+} release from this organelle is mediated by the IP3 receptor (IP3R)-type 3 and the Ryanodine receptors, we blocked each receptor in human NTM5 and GTM3 cells, two well-characterized TM cell lines with features similar to the
primary cultures.36 We used two IP3R inhibitors, 2-APB and Xe-C, and the ryanodine receptor inhibitor, dantrolene to measure changes in calcium release in the cells in the absence and presence of ROT. In Figure 5, we show that 2-APB and Xe-C were both capable of reducing ROT-induced increases \([\text{Ca}^{2+}]_c\), although 2-APB had a stronger effect. 2-APB decreased \([\text{Ca}^{2+}]_c\) by 2.63-fold \( (p < 0.05) \) whereas Xe-C decreased \([\text{Ca}^{2+}]_c\) by 1.23 fold \( (p < 0.05) \). Inhibition of the ryanodine receptor had little effect on the \([\text{Ca}^{2+}]_c\) in the cells (data not shown).

The results are expressed as the mean change in \([\text{Ca}^{2+}]_c\) from untreated NTM5 and GTM3 cells \( \pm \text{SE} \) of six different experiments \( (n = 6) \).

Based on these observations, we suggest that the IP3R-mediated release of calcium from SER stores is altered in the TM cells of patients with POAG.

### Relation of Elevated \([\text{Ca}^{2+}]_c\) in GTM cells to \([\text{Ca}^{2+}]_c\)-Induced Calcium Release from the Mitochondria (mCICR)

The release of calcium from SER stores can be triggered by several factors including CICR.59,60 We have already shown that the mitochondria in GTM cells produce high levels of ROS. Increased ROS production can cause opening of the MPTP with subsequent swelling and depolarization of the mitochondria, which, in turn, can cause a transient release of calcium into the cytosol.

In the present study, we determined whether mCICR plays a role in SER release of calcium in the GTM cells. The \([\text{Ca}^{2+}]_c\) in GTM cells was measured by flow cytometry after the cells were loaded with Rhod-2/AM. mCICR was measured over various time points after the TM cultures were treated with ROT or various concentrations of calcium or with simultaneous treatment with both ROT and \( \text{CaCl}_2 \). In Figure 6A, we show that ROT has no effect on \( \Delta \Psi_m \) in GTM cells in the presence of the \( \text{Ca}^{2+} \) chelator, BAPTA/AM. Flow cytometric studies showing changes in \( \Delta \Psi_m \) by TMRE fluorescence distribution after 30 minutes pretreatment with (A) BAPTA/AM \( (50 \mu M) \) and (B) EDTA \( (0.5 mM) \) of the two TM cultures before treatment with \( 5 \mu M \) ROT for 1 hour. The distribution profiles of cells stained with tetramethylrhodamine (TMRE) for 30 minutes show that ROT was ineffective in reducing \( \Delta \Psi_m \) when GTM cells were pretreated with BAPTA/AM. EDTA had only a minor effect on the ROT-induced decrease in \( \Delta \Psi_m \) in GTM cells.
that ROT triggered a \([Ca^{2+}]_m\) overload in the first 30 minutes in both GTM and NTM cells, although there was greater loading—1.99- \((\pm0.1)\), 2.55- \((\pm0.17)\), and 3.11-fold \((\pm0.16)\)—in the mitochondria of GTM cells after 5 \(\mu M\) ROT alone, 5 \(\mu M\) ROT+10 \(\mu M\) CaCl\(_2\), and 5 \(\mu M\) ROT+20 \(\mu M\) CaCl\(_2\), respectively. The increase was followed by a rapid decline in \([Ca^{2+}]_m\) in the presence of ROT (2.63 \(\pm0.17\)-fold) than Xe-C (1.23 \(\pm0.28\)-fold). The data suggest that the elevated \([Ca^{2+}]_m\) levels induced by ROT in GTM cells is partly dependent on the release of calcium from stores in the SER. The results are expressed as the mean change in \([Ca^{2+}]_m\), from untreated NTM5 and GTM3 cells \(\pm\) SE of six different experiments in NTM5 and GTM3 cells \(n=6\).

In addition, when the TM cells were pretreated for 30 minutes with 50 \(\mu M\) BAPTA/AM or 10 minutes with either 10 \(\mu M\) or 1 \(mM\) CaCl\(_2\), ROT did not decrease the \(\Delta\Psi_m\) in the GTM cells suggesting that ROT was ineffective in the absence of cytoplasmic calcium or after addition of low level of \(Ca^{2+}\) to the cells (Fig. 6B). Furthermore, removal of cytoplasmic calcium with BAPTA/AM resulted in an immediate increase in \(\Delta\psi_m\) and high levels of CaCl\(_2\) (1 \(mM\)) alone caused a decrease in the \(\Delta\psi_m\) in both in the cultures with stronger effects on the GTM cells (Fig. 6B). The results are expressed as mean change in \(\Delta\psi_m\) and \([Ca^{2+}]_m\) from untreated GTM cells \(\pm\) SE of six different experiments for each donor \(n=48\).

Taken together, the data imply that there is greater uptake and release of calcium in the mitochondria of GTM cells and that this is potentiated by increasing calcium levels. Incubating GTM cells in high Ca\(^{2+}\) concentration alone can also decrease the \(\Delta\psi_m\) below ROT induced levels, suggesting that the elevated cytoplasmic Ca\(^{2+}\) levels in GTM cells trigger mCICR.

**Expression of Cyclophilin D Proteins in GTM Cells**

In the final experiments we set out to understand a possible mechanism by which increased MPTP opening occurs in GTM cells by examining the expression of cyclophilin D. This protein is found in the mitochondrial membrane matrix and is a key component in the process of MPTP opening. It interacts with calcium, binds to the pore, and causes a conformational change in the structure of the MPTP, which results in its opening up the pore. \(^{24}\)

The experiments in Figure 9 indicate that there is an increased level of cyclophilin D in POAG TM cells compared with that in their nondiseased counterparts and that the expression of this protein can be further induced in these cells by either ROT or calcium treatments. Confirmation that cyclophilin D expression is associated with intracellular calcium levels was obtained when we chelated \([Ca^{2+}]_i\), with BAPTA/AM and found a reduction in the levels of cyclophilin D in the GTM cells. This observation is also supported by our studies shown in Figure 7, which indicated MPTP opening and results in Figure 8B.

In summary (Fig. 10), we propose that regulation of the mitochondria-SER calcium cycle is perturbed in GTM cells as a consequence of chronic or constitutive opening of MPTP.
stores, causing an elevation in \([Ca^{2+}]_{m}\), which, in turn, would trigger IP3R release from SER
ments. ROT triggered a \([Ca^{2+}]_{c}\) efflux with a decrease in \([Ca^{2+}]_{m}\) in GTM cells was dependent on mitochondria Ca\(^{2+}\) induced calcium release (mCICR). \([Ca^{2+}]_{m}\) in GTM cells was measured by flow cytometry after staining with rhod-2/AM before treatment with ROT. ROT did not decrease the level of \([Ca^{2+}]_{m}\) in the absence of cytoplasmic calcium or after addition of a low concentration of
mPTP, resulting in subsequent increase in \([Ca^{2+}]_{m}\) levels in GTM cells. These data indicate that partial or full inhibition of mPTP opening. Results are expressed as the mean change in \([Ca^{2+}]_{m}\), and \(\Delta[Ca^{2+}]_{m}\) and from untreated GTM cells ± SE of six different experiments for each donor (\(n = 48\)).

**DISCUSSION**

Abu-Amero et al.\(^ {30}\) reported a spectrum of mitochondrial abnormalities in patients with POAG. These include a decrease in mitochondrial respiratory activity and sequence alterations in the mtDNA. Their data provide some of the first evidence of a possible role for mitochondrial dysfunction in the pathogenesis of glaucoma. Their results were supported by our recent findings that there is a mitochondrial I defect in the TM cells of individuals with POAG that results in increased ROS produc-

**Figure 6.** ROT-induced \([Ca^{2+}]_{m}\) in GTM cells was dependent on mitochondria Ca\(^{2+}\) induced calcium release (mCICR). \([Ca^{2+}]_{m}\) in GTM cells was measured by flow cytometry after staining with rhod-2/AM. (A) mCICR was measured over the indicated time points and treatments. ROT triggered a \([Ca^{2+}]_{m}\) overload in the first 30 minutes in both GTM and NTM cells, although there was greater loading by 1.99- (±0.1), 2.55- (±0.17), and 3.11-fold (±0.16) in the mitochondria of GTM cells after 5 μM ROT alone, 5 μM ROT+10 μM CaCl\(_2\), and 5 μM ROT+20 μM CaCl\(_2\), respectively. This effect was followed by a rapid efflux with a decrease in \([Ca^{2+}]_{m}\) of 2.1- (±0.09), 3.54- (±0.07), and 4.51-fold (±0.07) over the influx peak and a decrease over the starting baseline by 5%, 28%, and 51%. High Ca\(^{2+}\) concentration alone can also decrease the \(\Delta[Ca^{2+}]_{m}\) similar to that seen when the cells are treated with ROT. (B) TM cells were pretreated with 50 μM BAPTA/AM for 30 minutes, or with either 10 μM CaCl\(_2\) or 1 mM CaCl\(_2\) for 10 minutes before treatment with ROT. ROT did not decrease the \(\Delta[Ca^{2+}]_{m}\) in GTM cells in the absence of cytoplasmic calcium or after addition of a low level of Ca\(^{2+}\). Results are expressed as the mean change in \(\Delta[Ca^{2+}]_{m}\) and \([Ca^{2+}]_{m}\) from untreated GTM cells ± SE of six different experiments for each donor (\(n = 48\)).

Constitutive opening of the pore is most likely regulated by the increased levels of cyclophilin D and calcium in the cells. This scenario would result in a decrease in \(\Delta[Ca^{2+}]_{m}\), followed by mCICR, which, in turn, would trigger IP3R release from SER stores, causing an elevation in \([Ca^{2+}]_{m}\), followed by an increase in \([Ca^{2+}]_{c}\). Such a nonphysiological feedback loop would certainly give rise to the chronic elevation in \([Ca^{2+}]_{c}\), \([Ca^{2+}]_{m}\), and the decreased \(\Delta[Ca^{2+}]_{m}\) that we observed in the GTM cells. Our experiments showing that these conditions can be exacerbated by ROT support our hypothesis.

**Figure 7.** Inhibitors of the MPTP had no effect on \([Ca^{2+}]_{m}\) levels but caused an increase in \(\Delta[Ca^{2+}]_{m}\) in TM cells exposed to ROT. Measurement of \(\Delta[Ca^{2+}]_{m}\) and \([Ca^{2+}]_{c}\), were performed by flow cytometry with TMRE and fluo-3/AM, respectively. (A) Pretreatment with the MPTP inhibitors Cys (3 μM) and ArA (50 μM) had little effect on the ROT-induced elevation of \([Ca^{2+}]_{m}\) levels in the GTM cells, suggesting that ROT's effect on \([Ca^{2+}]_{m}\) levels in the cells is not dependent on MPTP opening. (B) Pretreatment with the MPTP inhibitors, however, blocked the effect of ROT on \(\Delta[Ca^{2+}]_{m}\) and resulted in an increase in \(\Delta[Ca^{2+}]_{m}\) in TM cells, suggesting that POAG TM cells have increased occurrences of MPTP opening. Results are expressed as the mean change in \([Ca^{2+}]_{m}\), and \(\Delta[Ca^{2+}]_{m}\) from untreated TM cells ± SE of six different experiments for each donor (\(n = 48\)).

**Figure 8.** Blocking the mitochondrial uniporter blocks ROT-induced change in \([Ca^{2+}]_{m}\) levels in GTM cells. (A) Trifluoperazine, the Ca\(^{2+}\)/Na\(^{+}\) exchanger inhibitor, and ruthenium red (RR), the mitochondrial Ca\(^{2+}\) uniporter inhibitor, were added to TM cultures 30 minutes before ROT treatment in the combinations and concentrations indicated. The experiments show that only RR suppress ROT-induced loading of the \([Ca^{2+}]_{m}\) in GTM cells. (B) Similarly, pretreatment with RR reversed the effect of ROT on \(\Delta[Ca^{2+}]_{m}\), resulting in an almost immediate increase in \(\Delta[Ca^{2+}]_{m}\) in GTM cells. These data indicate that partial or full inhibition of mCICR by blocking the uniporter could result in parallel inhibition of MPTP, resulting in subsequent increase in \(\Delta[Ca^{2+}]_{m}\). Results are expressed as the mean change in \([Ca^{2+}]_{m}\) and \(\Delta[Ca^{2+}]_{m}\) from untreated TM cells ± SE of six different experiments for each donor (\(n = 48\)).
Ca2+ with POAG suggest a role for Ca2+ dysregulation in the pathogenesis of this disease. Recently, Xue et al.8 reported increased activity of the calcification marker ALP in glaucomatous TM and suggested that it might be indicative of an ongoing mineralization process during development of the disease. This process may cause the cells to lose their contractile abilities and would most likely result in increased IOP.

We further showed that the increased calcium level in glaucomatous TM cells is dependent on MPTP opening and the subsequent release of calcium from ER stores rather than uptake through the plasma membrane. The ER is a key site for intracellular calcium storage and is in close proximity to the mitochondria. These two organelles maintain intracellular calcium homeostasis. We found that chelating intracellular Ca2+ with BAPTA/AM caused an increase in the ∆Ψm of GTM cells, even in the presence of high extracellular Ca2+ concentrations, whereas EDTA had only a minor effect on ∆Ψm, implying that MPTP opening was predominantly controlled by [Ca2+]i.

Prolonged and massive influx of Ca2+ into the mitochondria can trigger a release of Ca2+ from the mitochondria (mCICR).23,64 In living cells, mCICR is triggered during IP3-induced Ca2+ mobilization and results in the amplification of the Ca2+ signals primarily emitted from the ER, causing more Ca2+ to be released from ER stores, MPTP opening, and cell degeneration.21,66 We found that inhibiting the mitochondria uniporter, a channel through which Ca2+ is effluxed from the mitochondria, blocks depolarization of ∆Ψm in GTM cells in the presence of ROT, suggesting that increased [Ca2+]m in POAG TM cells induced MPTP opening by mCICR. Though the precise mechanisms by which Ca2+ induces MPTP opening is still unclear, it is believed that this action is strictly dependent on the saturation of internal Ca2+-binding sites on the MPTP.62 It is, therefore, reasonable to assume that there is a Ca2+-threshold for MPTP opening.

Since mCICR is triggered during IP3-induced Ca2+ mobilization and results in the amplification of the Ca2+ signals primarily emitted from the ER,21 it is not surprising that we were able to block the increase in [Ca2+]i and [Ca2+]m in GTM cells with an IP3R inhibitor. The outcome of IP3R inhibition was lower [Ca2+]i and [Ca2+]m and an increase in ∆Ψm, confirming that MPTP opening in the GTM cells is provoked by a mitochondria-ER feedback loop. The increase in cyclophilin D that we observe in GTM cells is additional support for our hypothesis that there is frequent MPTP opening in POAG TM cells that sets off a cyclic cascade of events.

**Figure 9.** Levels of cyclophilin D were increased in GTM cells. RNA was extracted from NTM5 and GTM3 cells treated with 5 μM ROT, 1 mM CaCl2, or 50 μM BAPTA/AM for 24 hours. (A) RT- and (B) real-time PCR showed that the expression of cyclophilin D is higher in GTM3 cells by 1.5±fold (±0.08) compared to NTM5. Cyclophilin D expression was significantly increased in GTM3 cells after ROT and CaCl2 treatment by 2.3±fold (±1.03) and 2.05-fold (±0.12), respectively. There was a significant decrease (0.67 ± 0.09-fold) in GTM3 cells after BAPTA/AM treatment. The histogram represents relative abundance of cyclophilin D to GAPDH. Results are expressed as the mean change in mRNA level of cyclophilin D from untreated NTM ≥ SE of six different experiments in NTM5 and GTM3 cells (n=6). *P < 0.05. (C) Western blot analysis with the polyclonal antibodies against cyclophilin D (17.7 kDa) confirmed that there is higher cyclophilin D protein levels in GTM cells and that this can be increased by ROT and CaCl2 and reduced by BAPTA/AM.

**Figure 10.** Mitochondria ER Ca2+ regulation feedback loop in POAG TM cells and possible routes of inhibiting MPTP opening.

The high [Ca2+]i and [Ca2+]m in the TM cells of patients with POAG suggest a role for Ca2+ dysregulation in the pathologic course in glaucomatous TM.

In this study, we examined the levels of Ca2+ in TM cells from POAG and nondiseased aged-matched individuals and found that both [Ca2+]i and [Ca2+]m were higher in POAG TM and that these levels were augmented by ROT. In addition, we showed that elevated calcium levels in POAG TM cells were dependent on MPTP opening and IP3R-mediated release of Ca2+ from ER stores.

Ca2+ plays a central role in cell signaling14–16. Its concentration in the cellular environment changes in response to a range of signals that allow this ion to modulate cellular function. The mitochondria are not simple cellular power plants but participate in various intracellular processes including cellular Ca2+ signaling. They can modulate the amplitude and spatiotemporal organization of cytoplasmic Ca2+ signals because of their ability to rapidly accumulate and release Ca2+ into the cytosol.14,15,53,60 Mitochondria Ca2+ overloads leads to ROS overproduction, which in turn triggers MPTP opening and apoptotic mechanisms.54,63

The high [Ca2+]i and [Ca2+]m in the TM cells of patients with POAG suggest a role for Ca2+ dysregulation in the pathologic course in glaucomatous TM.
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From these observations, we propose that the mitochondrial respiratory chain complex I defect in POAG TM cells, which we have described elsewhere,36 sets up a feedback loop between the ER and the mitochondria that keeps intracellular Ca\(^{2+}\) levels high and \(\Delta\Psi\)m low in these cells (Fig. 10). This loop appears to be perpetually maintained in POAG TM cells by chronic MPTP opening and increased mCICR, suggesting that dysregulation of calcium levels underlie the progression of glaucoma.

Our findings have implications for pharmacologic inhibition of mCICR, MPTP opening, and cyclophilin D as possible mechanisms for regulation of Ca\(^{2+}\) thresholds in TM cells to prevent the degeneration of this tissue in glaucoma.

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