Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling in Human Trabecular Meshwork Cells

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PURPOSE. POAG is a progressive optic neuropathy that is currently the leading cause of irreversible blindness worldwide. While the underlying cause of POAG remains unclear, TGF-β2-dependent remodeling of the extracellular matrix (ECM) within the trabecular meshwork (TM) microenvironment is considered an early pathologic consequence associated with impaired aqueous humor (AH) outflow and elevated IOP. Early studies have also demonstrated markedly elevated levels of oxidative stress markers in AH from POAG patients along with altered expression of antioxidant defenses. Here, using cultured primary or transformed human TM cells, we investigated the role oxidative stress plays at regulating TGF-β2-mediated remodeling of the ECM.

METHODS. Primary or transformed (GTM3) human TM cells conditioned in serum-free media were incubated in the absence or presence of TGF-β2 and relative changes in intracellular reactive oxygen species (ROS) were measured using oxidation-sensitive fluorescent dyes CellROX green or 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA). TGF-β2-mediated changes in the content of connective tissue growth factor (CTGF) and collagen types 1 and 4 (COL1A1 and COL4A1) mRNA or collagen proteins I and IV isoforms were determined in the absence or presence of mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ) and quantified by quantitative PCR or by immunoblot and immunocytochemistry. Smad-dependent canonical signaling was determined by immunoblot, whereas Smad-dependent transcriptional activity was quantified using a Smad2/3-responsive SBE-luciferase reporter assay.

RESULTS. Primary or transformed human TM cells cultured in the presence of TGF-β2 (5 ng/mL; 2 hours) exhibited marked increases in CellROX or fluorescein fluorescence. Consistent with previous reports, challenging cultured human TM cells with TGF-β2 elicited measurable increases in regulated Smad2/3 signaling as well as increases in CTGF, COL1A1, and COL4A1 mRNA and collagen protein content. Pretreating human TM cells with mitochondrial-targeted antioxidants XJB-5-131 (10 μM) or MitoQ (10 nM) attenuated TGF-β2-mediated changes in Smad-dependent transcriptional activity.

CONCLUSIONS. The multifunctional profibrotic cytokine TGF-β2 elicits a marked increase in oxidative stress in human TM cells. Mitochondrial-targeted antioxidants attenuate TGF-β2-mediated changes in Smad-dependent transcriptional activity, including marked reductions in CTGF and collagen isoform gene and protein expression. These findings suggest that mitochondrial-targeted antioxidants, when delivered directly to the TM, exhibit potential as a novel strategy by which to slow the progression of TGF-β2-mediated remodeling of the ECM within the TM.

Keywords: TGF-β2, human, trabecular meshwork, mitochondria, oxidative stress, extracellular matrix

POAG is the leading cause of irreversible blindness worldwide. Currently affecting over 60 million individuals, this insidious age- and race-biased optic neuropathy is characterized by a gradual loss of retinal ganglion cell neurons and is projected to impact nearly 80 million people by the year 2020.1 The prevalence of POAG, the most common form of glaucoma, affects approximately 1.86%, or nearly 2 million individuals, 45 years of age and older in the United States.2 More recent data using the US Census Bureau’s national population projections (2014–2050) estimate the US population with glaucoma in 2010 at 2.7 million, 2.9 million in 2014, and will increase to 4.3 million and 5.5 million by the years 2032 and 2050, respectively.3 Several prospective, randomized, multicenter studies have identified elevated IOP as a primary risk factor for the onset and progression of POAG.4,6 The pathophysiology leading to
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Elevated IOP in affected patients remains poorly understood. However, cases of normal-tension glaucoma (~10%–15%, USA) suggest multiple etiologies. Maintenance of IOP is of critical importance to preserving the overall function and health of the retina. In healthy eyes, IOP is maintained through balanced production and outflow of aqueous humor (AH). In adult humans, the majority (>50%) of AH exits the eye through a conventional outflow pathway consisting of uveal and corneoscleral trabecular meshwork (TM), juxtacanalicular tissue (JCT), and Schlemm’s canal (SC). While the inner wall of SC is believed responsible for the bulk of AH outflow resistance, cells proximal to this region within the TM are thought to regulate AH outflow facility through (1) contraction and relaxation of their actin cytoskeleton, and (2) synthesis and secretion of appropriate extracellular matrix (ECM) components. Under pathologic conditions, aberrant contraction of TM cells and/or disproportionate accumulation of ECM components are believed to contribute to a decrease in AH outflow facility leading to elevated IOP.

Whereas the underlying cause of POAG remains unclear, multiple studies have demonstrated that TGF-β2, a multifunctional profibrotic cytokine that promotes ECM synthesis and deposition by TM cells, is markedly elevated in the AH of patients with POAG. Of the three TGF-β isoforms present in mammals, TGF-β2 has been associated with POAG most frequently. Experimental studies have shown that ex vivo perfusion of physiologic concentrations of biologically active TGF-β2 (5–10 ng/mL) through cultured porcine- or human-anterior segments significantly elevates IOP.

Application of TGF-β2 to human TM cells in culture enhances expression and secretion of a variety of ECM and ECM-related proteins, including plasminogen activator inhibitor-1, fibronectin, and tissue transglutaminase, while decreasing activity of matrix metalloproteinase-2. TGF-β2-facilitated induction of ECM synthesis and secretion is largely regulated by canonical Smad3/5-mediated signaling mechanisms. TGF-β2-mediated changes in actin stress fiber organization and contractility may also promote elevated IOP in part, through activation of noncanonical small monomeric Rho GTPase/Rho kinase signaling pathways. Alternatively, formation of cross-linked actin networks within TM cells may also promote elevation of IOP in response to TGF-β2. These, and other, studies underscore the concerted role multiple signaling pathways play in the regulation of TGF-β2-mediated increases in outflow resistance and IOP in POAG. Selective downstream targeting of individual pathways in isolation, however, is anticipated to only partially alter TGF-β2-mediated increases in outflow resistance.

Oxidative stress, implicated in the pathology of several neurodegenerative diseases, also plays a prominent early role in the pathophysiology of POAG. Numerous studies have documented (1) elevated levels of oxidative stress markers within the AH of affected POAG patients, (2) increased resistance to AH outflow by H2O2-induced TM degeneration, and (3) altered expression of antioxidant defenses (superoxide dismutase, catalase, and glutathione pathways) in glaucoma patients. The human TM appears to be exquisitely vulnerable to oxidative damage. Reactive oxygen species (ROS) are known to specifically affect the cellularity of the human TM. Selective oxidative damage to TM mitochondria may itself elicit TM cell dysfunction. Increased in ROS production associated with POAG may arise as a consequence of age-associated mitochondrial dysfunction.

Here, we examined whether mitochondrial-targeted antioxidants attenuate TGF-β2-mediated changes in Smad-dependent transcriptional activity, including marked reductions in CTGF, collagen transcriptional isoforms, and collagen protein expression.

Methods and Materials

Human Trabecular Meshwork Cell Culture

The use of human material in this study was approved by the Edward Hines Jr. VA Hospital institutional review board. Fresh corneoscleral rims were received in Optisol corneal storage medium from the Illinois Eye Bank (Chicago, IL, USA) at time of corneal transplant and primary human TM cell isolates were prepared using a collagenase-free procedure as we have previously described. The purity of primary TM cell cultures typically exceeded 95% as routinely determined by cell morphology. Primary isolates were passaged (1:5) using trypsin and subsequent primary (secondary normal) TM cell cultures were restricted to less than seven passages. To objectively validate the identity and stability of isolated TM cells, primary human TM cultures were routinely challenged with dexamethasone and analyzed for expression and release of myocilin. Briefly, TM cells were cultured to near confluency in 6-well plates with each well containing 2 mL of either low-glucose (5.6 mM) Dulbecco’s modified Eagle medium (DMEM) media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% amphotericin B (ThermoFisher Scientific, Ashville, NC, USA), and 1% TM cell growth supplement (ScienceCell Research Lab, Carlsbad, CA, USA), or with low-glucose MEM (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 5% adult bovine serum, essential and nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% amphotericin B. Media was replaced with an equal volume of fresh serum-free low-glucose media containing vehicle (0.25% dimethyl sulfoxide [DMSO]) or dexamethasone (95 nM). After 72 hours, an aliquot (1.2 mL) of conditioned cell culture media was centrifuged at 10000g × 5 minutes to remove nonadherent cells/debris and 1.0 mL of the resultant supernatant was treated with 10 μL of StrataClean Resin (Agilent Technologies, Santa Clara, CA, USA) to concentrate released myocilin. Resin-treated samples were gently mixed by repeated inversion x 20 minutes at 4°C and resin-captured myocilin collected by centrifugation (20,000g × 5 minutes). Aspirated supernatant was discarded, resin pellets were resuspended with 25 μL of Laemmli sample buffer and heated at 65°C x 10 minutes to elute captured myocilin. Samples were cleared by centrifugation (20,000g × 10 minutes) and an aliquot of the cleared supernatant was qualitatively analyzed by capillary electrophoresis (Wes; Protein Simple, San Carlos, CA, USA) or with low-glucose MEM (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 5% adult bovine serum, essential and nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% amphotericin B. Media was replaced with an equal volume of fresh serum-free low-glucose media containing vehicle (0.25% dimethyl sulfoxide [DMSO]) or dexamethasone (95 nM). After 72 hours, an aliquot (1.2 mL) of conditioned cell culture media was centrifuged at 10000g × 5 minutes to remove nonadherent cells/debris and 1.0 mL of the resultant supernatant was treated with 10 μL of StrataClean Resin (Agilent Technologies, Santa Clara, CA, USA) to concentrate released myocilin. Resin-treated samples were gently mixed by repeated inversion x 20 minutes at 4°C and resin-captured myocilin collected by centrifugation (20,000g × 5 minutes). Aspirated supernatant was discarded, resin pellets were resuspended with 25 μL of Laemmli sample buffer and heated at 65°C x 10 minutes to elute captured myocilin. Samples were cleared by centrifugation (20,000g × 10 minutes) and an aliquot of the cleared supernatant was qualitatively analyzed by capillary Western immuno-electrophoresis (Wes; Protein Simple, San Jose, CA, USA) for the presence of myocilin using a 1:50 dilution of mouse monoclonal anti-myocilin primary antibody (EMD Millipore, Temecula, CA, USA) and a ready-to-use horseradish peroxidase conjugated anti-mouse IgG secondary antibody preparation (Protein Simple, San Jose, CA, USA). A prominent doublet (arrows) migrating between 58 and 59 kDa was present in dexamethasone (lanes 4–6), but not vehicle (lanes 1–3), treated samples as shown in Figure 1.

SV40-transformed human TM cells (GTM3) were a generous gift from Alcon Laboratories (Fort Worth, TX, USA) and were cultured in low-glucose (5.6 mM) DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% amphotericin B. In contrast to passaged primary TM cells, conditioned media collected from transformed GTM3 cells and primary human TM cell isolates contained elevated levels of myocilin even in the absence of a dexamethasone challenge (data not shown). Primary and...
transformed human TM cell cultures were maintained at 37°C under a humidified atmosphere of 5% CO₂/95% air.

**Treatment**

Semiconfluent cultures of primary or transformed (GTM3) human TM cells were incubated overnight in serum-free low-glucose media unless noted otherwise. Recombinant human TGF-β2 (Bio-Techne Corporation, Minneapolis, MN, USA) was reconstituted with 4 mM HCl containing 0.1% BSA to a working concentration of 20 μg/mL and aliquots stored at -80°C until use. Stored aliquots were “reactivated” prior to use by diluting stocks 1:1 (vol/vol) with fresh 4 mM HCl-BSA to yield a final concentration of 10 μg/mL. Serum-starved cultures were treated in the absence (vehicle, 2 μM HCl) or presence of TGF-β2 (5 ng/mL). In some experiments, cells were pretreated with SB-431542 (10 μM, a TGF-β type I receptor [TGFβRI]/activin receptor-like kinase 5 [ALK-5] inhibitor) or with mitochondrial-targeted antioxidants XJB-5-131 (10 μM; generous gift from Peter Wipf) or mitoquinone mesylate (MitoQ, 10 nM; MedKoo Biosciences, Inc., Morrisville, NC, USA).

**Quantification of Oxidative Stress**

Intracellular ROS were qualitatively and quantitatively measured using oxidation-sensitive fluorogenic dyes CellROX green or 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA; ThermoFisher Scientific), respectively. For qualitative assessment, TM cells (15,000 per chamber) were cultured to approximately 60% confluency in 8-well Nunc Lab-Tek II chambered glass slides (ThermoFisher Scientific), serum-starved overnight, and subsequently treated as described above. CellROX green (5 μM) was added to the final 30 minutes of treatment. Dye-loaded cells were fixed by immersion in 3.7% buffered paraformaldehyde (pH 7.4) for 15 minutes at 23°C. Fixed cells were rinsed, air dried, and mounted with Fluoroshield containing 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Leica TCS SPE confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) and LAS-X imaging suite. To minimize photoactivation artifact, cells fields were imaged under identical confocal conditions using identical software settings. For quantitative assessment, TM cells were cultured in 96-well plates to near confluency and serum-starved overnight. Media was removed, and cells were preincubated × 30 minutes at 37°C with prewarmed Hanks’ balanced salt solution supplemented with carboxy-H2DCFDA (5 μM). Dye preloaded cells were subsequently incubated in fresh serum-free media for an additional 30 minutes at 37°C prior to treating as described above. The 2',7'-dichlorofluorescein diacetate fluorescence was quantified using a Labtech FLUOstar Optima luminometer (BMG Labtech, Cary, NC, USA) at 488-nm excitation/520-nm emission wavelengths.

**Real-Time RT-PCR**

Total RNA from TM cells was isolated using TRIzol reagent (ThermoFisher Scientific) and 1 μg was reverse-transcribed using iScript Supermix (Bio-Rad Laboratories, Hercules, CA, USA). cDNA sequences were amplified by real-time (iQ SYBR Green Supermix; Bio-Rad) quantitative PCR using a 48-well CFX Connect PCR detection system with published human-specific primer pairs (Table 1). Human-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pairs were used as a reference control. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. Reaction efficiencies were typically >90%. The endogenous expression of GAPDH was unaltered by drug treatment (data not shown). Relative fold changes in gene expression in each sample were therefore normalized to expressed levels of GAPDH.

**Immunoblot Analysis**

Human TM cells, treated as described above, were rinsed with ice-cold PBS and lysed by the addition of lysis buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X100, and 1% Nonidet P-40. Lysates were centrifuged at 13,000 × g for 15 minutes at 4°C, and the supernatant was collected and stored at -80°C. Protein concentration was determined using the Lowry assay. Equivalent amounts of protein per sample were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked overnight in 5% nonfat dry milk, and specific proteins were detected using commercially available primary and secondary antibodies. The specificity of the immunoblots was validated by stripping the membrane, reblocking, and reprobing with a different antibody.

**Table 1. qPCR Human Specific Primer Pairs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’Sense-3’ (Forward)</th>
<th>5’Anti-Sense-3’ (Reverse)</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>GCC TTA CCG ACT GGA AGA C</td>
<td>AGG AGG CGT TGT CAT TGG</td>
<td>143</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CTA AAG GCG AAC CTT GAT AT</td>
<td>TCC AGG AGC ACC AAC ATT AC</td>
<td>107</td>
</tr>
<tr>
<td>COL4A1</td>
<td>CGG GCC CTA AAG GAG ATA AAG</td>
<td>GAA CCT GGA AAC CCA GGA AT</td>
<td>115</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACC ACA GTC CAT GGC ATC AC</td>
<td>CCA CCA CCC TGT TGC TGT A</td>
<td>450</td>
</tr>
</tbody>
</table>
mM EDTA supplemented with a cocktail of protease (Roche Diagnostics Corp., Indianapolis, IN, USA) and phosphatase (Sigma Aldrich) inhibitors. Lysates were clarified by centrifugation (20,000g × 10 minutes at 4°C). To quantify secreted proteins, aliquots of conditioned media from treated cells were concentrated by centrifugal filtration (10-kDa cutoff; Amicon; Millipore, Bedford, MA, USA). Clarified cell lysates and concentrated conditioned media were stored at −80°C until use. Protein concentrations in clarified cell lysates and concentrated conditioned media were determined by the BCA method (Thermo Scientific) using BSA as a standard. Protein (10-μg protein per lane) in cell lysates or conditioned media were resolved by 4% to 20% SDS-PAGE gel electrophoresis either under reducing/denaturing (for Smads) or reducing (for collagens) conditions and transferred overnight onto nitrocellulose (Smads) or polyvinylidene difluoride (collagens) membranes as previously described. Membranes were blocked with 5% Carnation non-fat dried milk (Nestle, Arlington, VA, USA) in 10 mM Tris buffer (pH 7.4) containing 0.1% Tween 20 × 1 hour at 23°C and incubated overnight at 4°C in the presence of a 1:1000 dilution of rabbit monoclonal anti-Smad2 (clone 86F7), anti-phospho-Smad2 (clone 138D4; Ser465/467), anti-Smad3 (clone C67H9), anti-phospho-Smad3 (clone C25A9; Ser425/425) primary antibodies (Cell Signaling Technology; Danvers, MA, USA), or with a 1:1000 dilution of sheep polyclonal anti-collagen IV (ab6586; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-collagen I (ab90395; Abcam) or rabbit polyclonal anti-collagen 1 (AF6220-SP; R&D Systems, Minneapolis, MN, USA) or a 1:8000 dilution of rabbit polyclonal anti-collagen 1 (AF6220-SP; R&D Systems) secondary antibody, respectively. Immuno- blotted membranes were washed in Tris-Tween buffer and incubated × 1 hour at 23°C in the presence of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno- Research Labs, Inc., West Grove, PA, USA) or a 1:1000 dilution of horseradish peroxidase-conjugated donkey anti-sheep IgG (R&D Systems) secondary antibody, respectively. Immunostained membranes were visualized by enhanced chemiluminescence using Supersignal West Pico chemiluminescent substrate (ThermoScientific, Rockford, IL, USA).

Immunocytochemistry
Passaged human TM cells were cultured to near confluency in 8-well chambered slides, serum-starved overnight, and subsequently treated as described above. Treated TM cells were rinsed with ice-cold PBS (pH 7.4) and fixed by immersion in 4.0% buffered paraformaldehyde (pH 7.4) × 15 minutes at 23°C. Fixed cells were permeabilized with 0.05% Triton X-100 in PBS × 15 minutes at 23°C, blocked with 5% BSA in PBS (pH 7.4) for 1 hour at 23°C, and subsequently incubated overnight at 4°C in the presence of a 1:500 dilution of either mouse monoclonal anti-collagen I (ab90395; Abcam) or rabbit polyclonal anti-collagen IV (ab6586; Abcam) primary antibody, as indicated. Immunostained cells were washed in PBS, co-incubated for 1 hour at 23°C with a 1:100 dilution of either AlexaFluor 488-conjugated goat anti-mouse IgG or AlexaFluor 594-conjugated goat anti-rabbit IgG secondary antibody (Ther- moFisher Scientific), respectively, in the presence of DAPI (NucBlue; ThermoFisher Scientific). Washed cells were air dried, and mounted with Aqua PolyMount (PolySciences, Inc., Warrington, PA, USA). Cell immunofluorescence was captured using a Leica TCS SPE confocal microscope and imaging suite. Images were Z-stacked at 1-μm steps acquired from below the base of the cells to the top of the cells. Relative fluorescence intensities of representative immunostained fields were quantified using ImageJ software (http://image.nih.gov/ij/; provid- ed in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Measurements of Smad-Binding Element (SBE)-Luciferase Activity**
Transformed (GTM3) human TM cells were cultured in 96-well plates (20,000 cells per well) to near confluency and reverse transfected with 50 ng of luc2P/SBE/Hygro plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) according to the manufacturer’s protocol. The luc2P/SBE/Hygro plasmid is a pGL4.48 6052 bp vector containing Smad-binding response elements upstream of firefly luciferase 2P (1-hour half-life) gene. To control for potential variances in transfection efficiency, cells were co-transfected with thymidine kinase- Renilla luciferase. Transfected TM cells were subsequently cultured in serum-free media overnight and treated as described above. Following 24 hours of treatment, cells were lysed, and lysates were assayed for luciferase activity according to manufacturer’s protocol (Promega). Relative changes in luciferase activity were quantified by luminometry using a Labtech FLUOstar Optima luminometer (BMG LabTech, Inc., Cary, NC, USA).

**Quantification of Total TGF-β2 Content in Porcine Anterior Segment Perfusates**
Porcine globes were obtained fresh from a local abattoir within 1 to 2 hours of death by electrocution. Anterior segments were aseptically prepared within 4 to 6 hours of procurement and perfused with serum-free DMEM in pairs at a constant flow rate of 4.5 μL/min as previously reported. Following stabilization after a 24-hour washout period, pretreatment baseline aliquots (1.0 mL) of perfusates were collected and stored at −80°C. Stabilized porcine segments were subsequently perfused with vehicle (0.25% DMSO) or with XJB-5-131 (10 μM) for an additional 48 hours, at which time posttreatment aliquots (1.0 mL) of perfusates were collected and stored at −80°C. The content of total TGF-β2 protein released from and recovered in collected perfusates, as well as cell culture media, was determined using a commercially available ELISA kit (R&D Systems) according to the manufacturer’s protocol.

**Statistical Analysis**
Data are expressed as the mean ± SD of N observations unless noted otherwise. Statistical significance of parametric data was determined by Student’s t-test. Significance between multiple experimental groups was determined by one- or two-way ANOVA with a Dunnett’s multiple comparison post hoc analysis. In each case, P < 0.05 was considered statistically significant.

**RESULTS**
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2-Mediated Oxidative Stress
Primary or transformed human TM cells conditioned overnight in serum-free low glucose media responded robustly to a TGF-β2 challenge (5 ng/mL, 2 hours) by significantly increasing the intracellular content of ROS as quantified by CellROX green fluorescence (Fig. 2A). Pretreating TM cells with SB-431542 (10 μM, a TGF-β type 1 receptor inhibitor) or with mitochondriald-targeted antioxidants (10 nM, XJB-5-131 or MitoQ) completely prevented TGF-β2-mediated increases in intracellular ROS (Fig. 2A). Quantifying relative changes in intracellular levels of ROS using a distinctly different oxidation-sensitive fluorescent dye, carboxy-H2DCFDA, produced quantitatively similar findings. Carboxy-H2DCFDA preloaded TM cells re-
sponded to TGF-β2 (5 ng/mL, 2 hours) by increasing the content of intracellular ROS approximately 2-fold (Fig. 2B). Similar with that observed using CellROX, pretreating TM cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ) prevented TGF-β2–mediated increases in intracellular ROS (Fig. 2B).

Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Smad-Dependent Signaling

Consistent with previously reported studies, conditioning primary or transformed human TM cells overnight in serum-free low glucose media and subsequently challenging with TGF-β2 (5 ng/mL, 24 hours) elicited a marked (≥50-fold), statistically significant increase in phosphorylated Smad2 and phosphorylated Smad3 protein content as quantified by immunoblot (Figs. 3A, 3B). By comparison, co-treating TM cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ) markedly attenuated (≥90%) TGF-β2–mediated changes in Smad phosphorylation (Figs. 3A, 3B). To determine whether mitochondrial-targeted antioxidants were functionally effective at altering TGF-β2-mediated Smad-dependent transcriptional signaling, transformed TM cells were transiently transfected with a pGL4.48 construct containing a Smad-binding response element upstream of a firefly luciferase 2P gene. Following overnight conditioning in serum-free low glucose media, transfected cells treated with TGF-β2 (5 ng/mL, 16 hours) exhibited a marked more than 5-fold increase in luciferase activity (Fig. 3C). Pretreating transfected cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ) prevented TGF-β2–mediated increases in luciferase activity (Fig. 3C). By comparison, pretreating transfected TM cells with a water-soluble analog of vitamin E (Trolox, 100 μM) only marginally attenuated TGF-β2–mediated increases in luciferase activity (data not shown).

Mitochondrial-Targeted Antioxidants Attenuate TGF-β2–Mediated Increases in Collagens 1α1 and 4α1 mRNA Expression

In agreement with previous studies, TGF-β2 (5 ng/mL, 16 hours) also elicited a marked (≥5 to 25-fold) and statistically significant increase in the content of connective tissue growth factor (CTGF), collagen 1α1 (COL1A1), and collagen 4α1 (COL4A1) mRNA expression in conditioned primary or transformed human TM cells (Fig. 4). Pretreating TM cells with SB-431542 or with mitochondrial-targeted antioxidants (XJB-5-131 or MitoQ), however, prevented TGF-β2–mediated increases in CTGF, COL1A1, and COL4A1 mRNA content (Figs. 4A, 4B). In some cases, treating TM cells with SB-431542 or antioxidants alone elicited marked reductions in the baseline levels of CTGF, COL1A1, or COL4A1 mRNA content (data not shown).
FIGURE 3. Mitochondrially-targeted antioxidants attenuate TGF-β2 Smad-dependent signaling. Serum-starved confluent cultures of (A) primary or (B) transformed TM cells were pretreated (2 hour, 37°C) with vehicle (0.1% DMSO), SB-431542 (10 µM), XJB-5-131 (10 µM), or MitoQ (10 nM), and subsequently incubated for an additional 24 hours (37°C) in the absence or presence of TGF-β2 (5 ng/mL), as indicated. (A, B) Representative immunoblots of phospho- or total Smad 2/3 proteins present in lysates from treated TM cells. Data shown are the means ± SD (N = 3 separate experiments) of digitized band densities, expressed as a ratio of phosphorylated/total Smad proteins. (C) Relative lucinescence from Smad-binding element (SBE)-dependent luciferase activity. Data shown are the means ± SD (N = 4) from a single experiment, representative of two additional
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2–Mediated Increases in Collagens I and IV Subunit Isoform Protein Expression

Consistent with the observed increases in collagen isoform mRNA expression, primary TM cells conditioned overnight in serum-free, low-glucose media and subsequently challenged with TGF-β2 (5 ng/mL, 72 hours) elicited a marked (~1.5- to 2-fold) increase in collagen I and collagen IV protein expression as determined by immunocytochemistry (Fig. 5A). As we have previously reported, TGF-β2 also elicited a significant increase in phalloidin-positive filaments (F-actin stress fibers). TGF-β2–mediated increases in actin stress fiber formation and in collagens I and IV protein expression were prevented when assayed in the presence of SB-431542, XJB-5-131, or MitoQ (Figs. 5A). When assayed by immunoblot under reducing nondenaturing conditions, similar qualitative increases in the content of collagens I and IV proteins were seen in lysates of TM cells responding to a TGF-β2 challenge (Fig. 5B, lysates). Similarly, the content of collagens I and IV proteins present in concentrated TM cell culture media was markedly increased in response to a TGF-β2 challenge (Fig. 5B, media). Pretreating primary TM cells with SB-431542 prevented TGF-β2–mediated increases in collagen I and IV content in cell lysates and in culture media (Fig. 5B). While pretreating TM cells with XJB-5-131 or MitoQ only partially prevented TGF-β2–mediated increases in lysate collagen protein content, these antioxidants attenuated TGF-β2–mediated release of collagen protein isoforms into the culture media (Fig. 5B).

Mitochondrial-Targeted Antioxidants Alone Attenuate Endogenous Expression and Release of TGF-β2

As we have previously reported, human TM cells are capable of serving as an endogenous source of biologically active TGF-

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Mitochondrial-targeted antioxidants attenuate TGF-β2–mediated increases in CTGF, collagen 1α1, and collagen 4α1 mRNA content. Serum-starved confluent cultures of (A) primary or (B) transformed TM cells were pretreated (2 hours, 37°C) with vehicle (0.1% DMSO), SB-431542 (10 μM), XJB-5-131 (10 μM), or MitoQ (10 nM), and subsequently incubated for an additional 24 hours (37°C) in the absence or presence of TGF-β2 (5 ng/mL), as indicated. Shown are GAPDH-normalized changes in CTGF, collagen 1α1, and collagen 4α1 mRNA content from treated (A) primary or (B) transformed TM cells, as indicated. Data are the means ± SD (N = 4) from a single experiment. *P < 0.05; **P < 0.01, compared with vehicle controls; one-way ANOVA with Dunnett’s post hoc analysis. In all but one case, TGF-β2–mediated responses from cells pretreated with SB-431542 or with antioxidants were not significantly different from vehicle-treated controls. Pretreatment with mitoquinone was not as effective as XJB-5-131 at attenuating TGF-β2–mediated changes in Smad2 phosphorylation.
Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Signaling

**DISCUSSION**

In this study, the multifunctional profibrotic cytokine TGF-β2 is shown to elicit a marked increase in intracellular ROS in both transformed and primary human TM cells, as quantified using two distinct oxidation-sensitive fluorogenic dyes. Pretreating TM cells with mitochondrial-targeted antioxidants attenuated TGF-β2-mediated changes in ROS and in Smad-dependent canonic signaling, including marked reductions in actin stress fiber formation, as well as reductions in CTGF and collagen isoform gene and protein expression. Collectively, these findings suggest that mitochondrial-targeted antioxidants exhibit potential as a novel strategy by which to attenuate TGF-β2–mediated remodeling of the ECM within the TM.

Numerous clinical and experimental studies now support a pivotal role for oxidative stress in the early development and progression of several retinal neurodegenerative diseases, including POAG. Produced as a natural byproduct of enzymatic NADPH oxidase (NOX) and oxidative metabolism, highly reactive superoxide anions (O₂⁻) and hydroxyl (OH) free radicals are largely generated in excess amounts within the mitochondrial respiratory chain as a consequence of compromised or otherwise altered cellular respiration. Membrane permeant hydrogen peroxides can further react with the reduced form of intracellular iron (Fe²⁺) to generate additional amounts hydroxyl free radicals via the Fenton reaction. These reactive oxidants are thought to exert their deleterious effects intracellularly by chemically altering nucleic acids, polyunsaturated lipids, proteins, as well as carbohydrates.

Oxidative stress, in general, is defined as an imbalance between natural intracellular antioxidant defense mechanisms and metabolically generated ROS and has been reported to elicit pronounced functional and morphologic impairments in retinal pigment epithelial cells, endothelial cells, and retinal ganglion cell neurons. Cells of the TM are no exception and...
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have proven to be exquisitely vulnerable to oxidative injury. Several lines of additional evidence suggest that POAG may arise as a consequence of age-related mitochondrial dysfunction. A precedence for mitochondrial-dependent cellular energy dysfunction is not, however, unique to POAG. Mitochondrial-dependent pathologies are now overwhelmingly implicated in the genesis of numerous ocular and nonocular neurodegenerative disorders.

Profibrotic disorders involving excessive TGF-β signaling are, similarly, not unique to POAG. Pulmonary fibrosis, for example, is one such disease where oxidative stress and aberrant TGF-β signaling, two seemingly unrelated pathogenic mechanisms, have been causally linked. Mitochondrial-generated ROS have been shown to play a penultimate role in TGF-β-mediated pulmonary fibrosis. Advancements from pulmonary and other fields collectively demonstrate that (1) TGF-β alone induces the generation of mitochondrial ROS from complex III of the electron transport chain, (2) mitochondrial generated ROS are required for TGF-β-induced gene expression downstream of TGF-βRI receptor activation, (3) upstream of Smad2/3 phosphorylation and nuclear translocation, (4) TGF-β-induced transcription of NADPH oxidase 4 requires mitochondrial generated ROS, thereby establishing a feed-forward reciprocal loop leading to increased production of intracellular ROS, (5) fibroblasts from patients with lung fibrosis produce more mitochondrial ROS and a higher level of profibrotic gene expression in response to TGF-β than lung fibroblasts from normal donors, and (6) blocking mitochondrial ROS generation with a mitochondrial-targeted antioxidants markedly attenuates TGF-β-induced profibrotic gene expression.

What remains to be established, and is addressed in part by this study, is whether a similar feed-forward reciprocal relationship between TGF-β2 signaling and mitochondrial-generated ROS occurs in human TM cells. We and others have previously reported that cultured human TM cells respond robustly to a TGF-β2 challenge by promoting F-actin stress fiber formation and by eliciting the expression of ECM components. Human TM cells are also capable of constitutively expressing and releasing biologically active TGF-β2, serving as a localized source of this profibrotic cytokine. Consistent with the reported POAG-associated pathogenicity of TGF-β2, we now show that this cytokine is capable at physiologic levels of eliciting a robust and sustained increase in oxidative stress in human TM cells. In agreement with previous studies, TGF-β2-mediated production of oxygen free radicals did not appear to alter cultured TM cell viability as quantified by MTT and LDH release assays (data not shown). TGF-β2 did, however, elicit measurable increases in TGF-β type I receptor (Alk5)-dependent phosphorylation of regulated Smads 2/3 and Smad-binding element activity, consistent with activation of the canonical signaling pathway. Pretreating cultured TM cells with mitochondrial-targeted antioxidants not only prevented TGF-β2-mediated increases in oxidative stress, but also unexpectedly attenuated canonical signaling by this cytokine. The mechanism(s) by which oxidative stress enhances TGF-β2 canonic signaling remains poorly understood but may involve inhibition of redox sensitive protein phosphatases (PP1, PP2A, or PTEN). These data raise the intriguing possibility that TGF-β2-generated intracellular ROS enable or facilitate TGF-β2 canonic signaling in human TM cells.

The functional relevance of this observation is hallmarked by the ability of mitochondrial-targeted antioxidants to significantly attenuate TGF-β2-mediated increases in F-actin stress fiber formation and TGF-β2-mediated increases in

**Table 2. Mitochondrial-targeted Antioxidant XJB-5-131 Reduces Total Endogenous TGF-β2 Content Recovered in Porcine Anterior Segment Perfusate**

<table>
<thead>
<tr>
<th>Pretreatment Perfusate</th>
<th>Posttreatment Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>XJB-5-131</td>
</tr>
<tr>
<td>Segment Pair 1</td>
<td>Segment Pair 2</td>
</tr>
<tr>
<td>Mean</td>
<td>Mean</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>XJB-5-131</th>
<th>Vehicle</th>
<th>XJB-5-131</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment Pair 1</td>
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<td>501.3</td>
<td>657.7</td>
<td>339.5</td>
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<tr>
<td>Segment Pair 2</td>
<td>498.3</td>
<td>407.1</td>
<td>514.0</td>
<td>285.7</td>
</tr>
<tr>
<td>Mean</td>
<td>517.3</td>
<td>454.2</td>
<td>585.9</td>
<td>512.6</td>
</tr>
</tbody>
</table>

Pretreatment perfusate (baseline) was collected from stabilized porcine anterior segments following a 24-hour washout period. Posttreatment perfusate was collected 48 hours following perfusion with vehicle (0.25% DMSO) or XJB-5-131 (25 μM). Absolute amounts of total TGF-β2 released by segments and recovered in 1-mL aliquots of pre- or posttreatment perfusate was quantified ELISA. Normalization to total protein concentration did not alter these findings.
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References


expression of CTGF and collagens I and IV ECM constituents. Given the well-established ability of TGF-β2 to increase IOP, presumably through remodeling of the ECM, these data would seem to support a central role of mitochondrial-generated ROS in TGF-β2-mediated decreases in outflow facility and increased IOP in POAG. In agreement with this thesis, the mitochondrial-targeted antioxidant XJB-5-131 is shown to significantly attenuate the endogenous expression and release of TGF-β2 from transformed human TM cells. In addition, perfusing stabilized porcine anterior segments with XJB-5-131 reduced the levels of total TGF-β2 present in segment perfusates. These data not only support endogenous expression and release of TGF-β2 within the TM but also suggests that it is possible to alter, in vivo, endogenous TM levels of this profibrotic cytokine.

Whether mitochondrial-generated ROS are causal to, or a consequence of, the disease process remains unclear. If causal, one may anticipate a beneficial clinical effect of supplementary antioxidant therapy on development/progression of POAG. Epidemiologic studies conducted evaluating the impact of supplementary intake of antioxidants (vitamins A, C, or E) at altering the risk or prevalence of POAG has, however, largely been unsupportive. An early study by Kang et al.59 failed to observe any strong associations between vitamin A consumption and the risk of POAG. These investigators did report a statistically significant, albeit isolated, protective effect in the third quintile of total vitamin C intake on odds risk of glaucoma. A study by Coleman et al.61 similarly failed to find an association between glaucoma and vitamin nutrients. Interestingly, in the Coleman et al. study, consumption of certain foods, such as collards, kale, and carrots, appeared to be protective against glaucoma, unrelated to the intake of vitamins A, C, or E. A more recent study by Wang et al.62 also failed to find an association between supplementary consumption of vitamins A or E and glaucoma prevalence. In this latter study, however, vitamin C supplementation was found to be associated with decreased odds ratio (0.47, 95%CI 0.23–0.97) for glaucoma. No significant differences in ascorbic acid content, however, were present in AH of POAG patients compared with AH from healthy controls.62 In contrast, ascorbic acid levels present in AH of patients with exfoliation syndrome or POAG were substantially lower than in AH of patients with cataract.35 In some63–65 (but not all66–67) studies, administration of ascorbic acid to glaucoma patients markedly reduced IOP.

While these and other related clinical trials would argue against the effectiveness of supplementary antioxidant therapy as an effective adjuvant for the management of POAG, a potential limitation of these trials may be attributed to (1) the route of antioxidant administration rather than a lack of antioxidant effect, and/or (2) the type of antioxidants used as previous studies have shown that mitochondrial-targeted antioxidants offer greater protection against oxidative stress compared with nontargeted antioxidants.68–69 Based on the findings from the current in vitro study, we propose that mitochondrial-targeted antioxidants might serve as a useful adjunct to conventional proven IOP-lowering therapies. Targeted delivery of antioxidants with the use of nanotechnology is emerging as a viable alternative strategy by which to deliver such adjuncts directly to the site of greatest resistance (the JCT) and improve therapeutic efficacy in POAG.

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55. Cucoranu I, Clempus R, Dikalova A, et al. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differen-
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