Aspirin-Triggered Lipoxin A4 Attenuation of TM Cell Profibrotic Responses

**Scientific Abstract**
Glaucoma is the leading cause of irreversible blindness worldwide and is a substantial source of visual impairment to millions of individuals. Glaucoma most commonly manifests as open-angle glaucoma (POAG), in which a progressive loss of retinal ganglion cell (RGC) neurons in the setting of open anterior chamber angles is observed. The indolent nature of this disease underscores the importance of early detection and intervention to arrest further visual impairment. Current treatment paradigms involve non-selective therapeutic strategies that serve to reduce intraocular pressure (IOP), a known POAG risk factor. The development of targeted therapies that address the cause of POAG is crucial to advance the management of affected individuals.

Although the cause of POAG remains unclear, oxidative stress is an early pathologic hallmark of this age-associated disorder. Reactive oxygen species (ROS) are known to specifically affect the cellularity of the trabecular meshwork (TM), the primary anatomical site of aqueous humor (AH) outflow in adults. TGF-β2, a potent profibrotic cytokine strongly implicated in the pathogenesis of POAG, elicits marked increases in TM cell ROS.

A well-established consequence of sustained oxidative stress that is often overlooked in POAG is chronic smoldering inflammation. While previous studies have focused on the optic nerve/optic nerve head as the major site for inflammatory injury in POAG, we propose in this ISPB pre-clinical application that the TM may similarly experience age (ROS)-associated chronic inflammatory complications.

Acute inflammatory responses elicited by TGF-β2 mediated oxidative stress typically resolve on their own in response to locally produced lipid autocoids: lipoxins and resolvins. However, exposure to sustained oxidative stress may adversely alter endogenous operative resolution programs within the TM itself ultimately exacerbating TGF-β2 mediated signalling culminating in reduced outflow facility with subsequent elevation of IOP.

In this pre-clinical study, we hypothesize that exogenously applied lipid autocoids will attenuate TGF-β2 mediated increases in TM cell oxidative stress induced profibrotic responses. The hypothesis of this study will be tested with the following Specific Aim using established in vitro experimental approaches.

We will determine whether pre-conditioning human primary TM cells with lipid autocoids will alter endogenous TGF-β2 expression or TGF-β2 mediated signalling responses as quantified using established methodology including ELISA, RT-PCR, immunoblot, and immunocytochemistry.

By demonstrating that lipid autocoids exhibit a greater capacity to resolve TGF-β2 mediated oxidative stress, findings from this study may lead to a new generation of lipid-soluble therapeutic agents that specifically target the TM for the management of POAG.

**Lay Abstract**
Primary open angle glaucoma (POAG) is the leading cause of irreversible blindness worldwide and is a substantial source of visual impairment to millions of individuals. Although the cause of POAG remains unclear, oxidative stress is an early pathologic hallmark of this age-associated disorder. A well-established consequence of sustained oxidative stress is chronic smoldering inflammation. In young healthy individuals, inflammatory responses to oxidative stress resolve on their own. In older individuals, however, the ability to resolve inflammatory responses is compromised. We speculate that this may be an early cause of POAG. In this study, we will determine whether restoring this resolution program can attenuate the production of harmful oxidative free radicals in cultured trabecular meshwork cells. Findings from this study may lead to a new generation of therapeutic agents that facilitate in the management of POAG.
RESEARCH PROPOSAL

Statement of Purpose
Primary open angle glaucoma (POAG) remains a leading cause of irreversible blindness worldwide. Currently affecting over 70 million individuals, this insidious age-associated optic neuropathy is characterized by a gradual loss of retinal ganglion cells (RGC). Despite being an extraordinarily significant socioeconomic burden, the treatment of patients with POAG remains limited and palliative. Current treatment options are restricted to non-specific interventions aimed at lowering aberrantly elevated intraocular pressure (IOP), a poorly understood hallmark of POAG. For many glaucomatous patients, however, pharmacological and surgical management of IOP remains clinically refractive. The development of targeted therapeutic strategies directed at the cause of elevated IOP is critical for the advanced management of glaucomatous patients.

Whereas the underlying cause of POAG remains unclear, multiple studies have demonstrated that TGF-β2, a multifunctional profibrotic cytokine that promotes synthesis and deposition of extracellular matrix (ECM) proteins within the trabecular meshwork (TM), is markedly elevated in the aqueous humor (AH) of affected POAG patients. While the development of selective TGF-β2 downstream signaling pathway inhibitors continues to be the focus of intense investigations, disruption of constitutive TGF-β2 expression and release from the TM represents an under-explored therapeutic strategy for the management of IOP in POAG. Oxidative stress is a key component in the pathophysiology of POAG. The human TM is exquisitely vulnerable to oxidative damage. Recent experimental findings from our lab demonstrate that physiological concentrations of TGF-β2 elicit marked increases in the expression and release of ECM proteins from TM cells in direct response to TGF-β2 induced oxidative stress. Consistent with this thesis, pre-treating TM cells with mitochondrial-targeted antioxidants markedly attenuated TGF-β induced profibrotic gene expression.

Inflammation plays a penultimate underlying role in many neurodegenerative diseases, including glaucomatous neuropathy. It is also well-established that oxidative stress, implicated in the pathogenesis of POAG, leads to chronic inflammation. While previous studies have focused on the optic nerve/optic nerve head as the major site for inflammatory injury in POAG, we propose in this ISPB pre-clinical application that the TM may similarly experience age-associated chronic inflammatory complications. Acute inflammatory responses develop and actively resolve to re-establish homeostasis in response to locally produced lipid autocoids: lipoxins and resolvins. Transition from acute (beneficial) to chronic (harmful) inflammation is believed to result from a decline or complete loss of endogenously operative pro-resolution programs. While cyclooxygenase (prostaglandins) and lipoxygenase (leukotrienes)-derived arachidonic acid (20:4 ω6) metabolites have well-established roles in promoting inflammation, it is now clear that anti-inflammatory/pro-resolving (ω6 lipoxins and ω3 resolvins) lipid mediators are endogenously produced in a temporal and spatial manner to actively turn off localized inflammation. Lipoxin A4 is a sequential 12/15-lipoxygenase metabolite of arachidonic acid that atypically exhibits pro-resolving properties. In the presence of aspirin, the endoperoxide synthase COX-2 has been shown to catalyze the synthesis of highly potent pro-resolving 15-epimer of lipoxin termed aspirin-triggered lipoxin A4. By comparison, 15-lipoxygenase metabolites of the ω3 fatty acids eicosapentaenoic acid and docosahexaenoic acid give rise to the E- and D-series of resolvins, respectively, which have been previously shown to play a role in the resolution phase of acute inflammation of many disease states. Of particular relevance to this application are in vitro studies with isolated endothelial cells showing that inflammation ensues, in part, due to the loss of operative pro-resolution programs. It is not unreasonable to anticipate that if similar programs exist within the TM, an age (oxidative stress)-associated decline or loss of such an operative pro-resolution program(s) would be anticipated to exacerbate TGF-β2-mediated signaling within the TM in a vicious feedback cycle.

Hypothesis: Exogenously applied lipid autocoids will attenuate TGF-β mediated increases in TM cell oxidative stress induced profibrotic responses.

Specific Objective: We will determine whether pre-conditioning human primary TM cells with lipid autocoids will alter endogenous TGF-β2 expression or TGF-β2 mediated signalling responses as quantified using established methodology including ELISA, RT-PCR, immunoblot, and immunocytochemistry.

Clinical Relevance: Glaucoma remains a leading cause of irreversible blindness. Current treatment options are restricted to non-specific interventions aimed at lowering IOP. For many glaucomatous patients, however, management of IOP remains clinically refractive. The development of therapeutic strategies directed at the cause of elevated IOP is critical for the advanced management of glaucomatous patients. By demonstrating that lipid autocoids exhibit a greater capacity to resolve TGF-β2 mediated oxidative stress in cultured human TM cells, findings from this study may lead to a new generation of lipid-soluble therapeutic agents that specifically target the TM for the management of POAG.
**Preliminary Data**

**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 (Figure 1). Pretreating TM cells with SB-431542 (10 µM, a TGF-β type I receptor inhibitor) or with mitochondrial-targeted antioxidants (10 nM, XJB-5-131 or MitoQ) completely prevented TGF-β2 mediated increases in intracellular ROS (Figure 1).²

**Figure 1.** Mitochondrially-targeted antioxidants attenuate TGF-β2 induction of oxidative stress. Serum-starved confluent cultures of primary or transformed TM cells were pre-treated (2h, 37°C) with vehicle (0.1% DMSO), SB-431542 (10 µM), XJB-5-131 (10 nM), or MitoQ (10 nM) and subsequently incubated for an additional 2h (37°C) in the absence or presence of TGF-β2 (5 ng/ml), as indicated. Shown (left panels) are representative confocal micrographs of CellROX Green fluorescent images of DAPI counterstained cells. Bar, 100 µM. Right panels, Image J quantified CellROX Green fluorescent fields. Data shown are the means ± SD (N=4) of vehicle-normalized images. *, p < 0.05; **p < 0.01, compared with vehicle controls; one-way ANOVA with Dunnett’s post-hoc analysis.

**Mitochondrial-Targeted Antioxidants Attenuate TGF-β2 Mediated Increases in Collagens I and IV Subunit Isoform Protein Expression**

Consistent with the observed increases in collagen isofrom 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 (Figure 2). 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 (Figure 2).²

**Aspirin-Triggered Lipoxin A4 Attenuates TGF-β1 Mediated Increases in Collagen I Protein Release**

In support of our proposed hypothesis and consistent with the role of lipid autocoids acting at the level of the trabecular meshwork, we present un-published preliminary findings demonstrating the ability of epi-lipoxin A4 (100 nM) to attenuate TGF-β1 signaling in transformed TM cells (Figure 3). These findings, while preliminary, demonstrate study feasibility and provide the first evidence for a protective role of lipid autocoids against TGF-β1 mediated changes in TM extracellular matrix environment.
Figure 2. Mitochondrial-targeted antioxidants attenuate TGF-β2 mediated increases in collagen I and collagen IV protein isoforms. Serum-starved confluent cultures of primary TM cells were pre-treated (2h, 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 72h (37°C) in the absence or presence of TGF-β2 (5 ng/ml), as indicated. Shown (left panels) are representative fluorescent confocal micrographs of treated TM cells immunostained for the presence of collagen I (COLI, green) or collagen IV (COLIV, red) protein isoforms or phalloidin-positive filaments (F-actin, green) and DAPI counterstained. Bar, 100 µM. Right panels, Image J quantified immunofluorescent fields. Data shown are the means ± SD (N = 8) of vehicle-normalized images. **, p < 0.01, compared with vehicle controls; one-way ANOVA with Dunnett’s post-hoc analysis.

Figure 3. Epi-Lipoxin A4 attenuates TGF-β1 mediated increases in collagen I protein release. Serum-starved confluent cultures of transformed TM cells were treated with vehicle (0.1% ethanol), TGF-β1 (5 ng/ml), or TGF-β1 (5 ng/ml) plus Epi-Lipoxin A4 (100 nM) and cultured overnight at 37°C, as indicated. Cell culture media was collected, clarified, and treated with StrataClean resin to concentrate released proteins. Protein concentrates were resolved by capillary electrophoresis and resolved proteins immunostained using sheep polyclonal anti-collagen Iα1 primary and horseradish peroxidase-conjugated donkey anti-sheep IgG secondary antibody. Immunoblot shown is from a single experiment of triplicate determinations. A single band migrating at a molecular mass of 230-kDa was observed and is most likely a cross-linked species of collagen alpha monomers. Biotinylated molecular weight markers are shown for comparison.
Research Plan

Study Design and Methods

This one-year pre-clinical study is designed to determine whether lipid autocoids will alter endogenous TGF-β2 expression or TGF-β2 mediated signalling responses in cultured human primary TM cells. To our knowledge, we are the first to explore the experimental potential of lipid autocoids as a novel strategy for the management of POAG.

Human TM cell culture: We are in a unique position to continue to utilize resources on the Hines/Loyola campus (Department of Ophthalmology) that enables us to procure, harvest, and culture primary human TM cells. Primary human TM cells are prepared as previously described. Individual primary TM cell lines are restricted to less than seven passages. In all cases, TM cell viability after experimental treatment is determined by Trypan blue dye exclusion. TM cell viability is typically >90%. To address realistic limitations on primary TM cell availability and passage restrictions, all experiments will be first performed using transformed human TM cells so as not to impede research progress. Simian virus 40 (SV40)-transformed human TM cells will be maintained in culture as previously described. All seminal observations will be repeated using primary human TM cells to confirm relevance and minimize observations unique to the transformation phenotype.

Specific Objective: We will determine whether pre-conditioning human primary TM cells with lipid autocoids will alter endogenous TGF-β2 expression or TGF-β2 mediated signalling responses as quantified using established methodology including ELISA, RT-PCR, immunoblot, and immunocytochemistry. Previously, we have shown that quiescent cultures of primary or transformed human TM cells will be pre-treated with 0, 10, 50, or 100 nM of control (vehicle) or commercially available lipid autocoids (lipoxin A4, epi-lipoxin A4, resolin E1) for various times (0, 1, 8, 16, 24h) prior to (protective) or following (reversal) challenge with TGF-β2 (5 ng/ml). To exclude any non-specific toxic effects of these treatments, cell viability will be routinely monitored by either Trypan blue dye exclusion, release of LDH into the culture medium, or by MTT assay as previously described. Relative changes in TGF-β2 expression/release or TGF-β2 mediated changes in signaling (Smads) or target gene expression, including proET-1, CTGF, α-smooth muscle actin, and ECM components (collagens I, IV, and VI) will be quantified by ELISA, immunoblot, immunocytochemistry, qRT-PCR as we have previously described.

Methodology: ELISA: The absolute content of total and biologically-active TGF-β2 protein secreted from treated cells will be quantified using commercially available human specific ELISA kits according to manufacturer’s instructions as we have previously reported. Immuno blot: Treated cells are lysed by scraping into ice-cold deionized water supplemented with a commercial cocktail of protease and phosphatase inhibitors and prepared immunoblots stained using commercially available monoclonal or polyclonal primary antibody (Millipore; Santa Cruz; Cell Signaling; typically 1:1000 dilution) and goat anti-species IgG horseradish peroxidase–conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc; 1:10,000 dilution). To determine transcriptional- or posttranscriptional dependent changes in TGF-β2 mediated changes in target gene expression, some cultures will be pre-treated with actinomycin D (0.5 µg/ml) or cycloheximide (5 µM), respectively, prior to treatment. To exclude any non-specific toxic effects of these treatments, cell viability will be routinely monitored by either Trypan blue dye exclusion, release of LDH into the culture medium, or by MTT assay as previously described. To determine which ECM proteins are most affected by treatment, prepared immunoblots are stripped and stained with isoform-selective primary antibodies where possible. In all cases, immunoblots are stripped and stained with a 1:10,000 dilution of rabbit anti-GAPDH polyclonal (Trevigen) primary antibody as a protein loading control. Immunostained proteins are visualized by ECL. Relative changes in total protein content are quantified by densitometry. Immunocytochemistry: Human primary or transformed TM cells are cultured to semi-confluency on chambered coverslips and treated as described above. Cells are fixed for 15 min at 23°C by immersion in 0.1M phosphate-buffered 4% paraformaldehyde and relative changes in TGF-β2 expression or TGF-β2 mediated changes in target gene expression, including CTGF, α-smooth muscle actin, and ECM components (collagens I, IV, and VI) are semi-quantified by immunostaining using commercially available monoclonal or polyclonal primary antibody (Millipore; Santa Cruz; Cell Signaling; typically 1:100 dilution) and goat anti-species IgG Alex Fluor 594–conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc; 1:2,500 dilution). Immunocytochemical findings will be semi-quantified using Image J as we have previously described. qRT-PCR: Treated cells are harvested by scraping and total RNA is extracted using TRIzol reagent (Invitrogen) and 5 µg reverse-transcribed (Super Script III First Strand Synthesis system; Invitrogen). Human Col1A1-, Col1A2-, Col4A1-, Col6A1-, and GAPDH-specific cDNA sequences are amplified by real-time (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) quantitative RT-PCR using a PCR detection system (CFX Connect, Bio-Rad). Human-specific
oligonucleotide primer pairs based on published sequences will be commercially prepared (ThermoFisher). For each sample, the specificity of the real-time reaction product will be determined by melting curve analysis. Where needed, amplicon size will be determined by agarose gel electrophoresis to verify product authenticity. In all cases, GAPDH primers are used as a reference control. Once confirmed unaffected by autocoid treatment, relative fold-changes in gene expression in each sample will be normalized to expressed levels of GAPDH.

**Statistical Plan**

Proposed experiments using transformed human TM cells will be performed in triplicate and repeated two additional times for statistical significance. Experiments using primary human TM cells will be performed in duplicate and repeated two additional times for statistical significance. Data are expressed as the mean ± SD of N observations. Statistical significance of single group parametric data will be determined by Student’s t-test. Significance between multiple experimental groups is determined by one- or two-way ANOVA with a Dunnett’s multiple comparison post hoc analysis. In each case, P < 0.05 will be considered statistically significant.

**Anticipated Results and Alternative Strategies**

Based on existing literature, we anticipate that pre-treating human TM cells with lipid autocoids will elicit protection against TGF-β2 mediated increases in oxidative stress. We further expect that adding lipoxin/resolving autocoids to TGF-β2-challenged cells will effectively truncate oxidative stress-mediated changes. Autocoids are anticipated to protect against relative changes in TGF-β2 expression or TGF-β2 mediated changes in target gene expression. Differential responses between lipoxin and resolvin autocoids would highlight distinctive metabolic pathways, revealing important mechanistic contributions. An emerging body of evidence now supports epigenetic modifications induced by oxidative stress, including hypomethylation of CpG islands, modification of histone proteins, or microRNAs. Indeed, endogenous TGF-β2 mRNA and protein expression in TM cells is modestly regulated by miR-29b, possibly by regulating expression of Rho family of GTPases. Another possibility involves TGF-β2-mediated changes in the relative expression of protective antioxidant enzymes, including catalase, SOD, and/or glutathione peroxidase. Lipid autocoids may protect against a TGF-β2 challenge by restoring balance to these defenses. Beyond the scope of the present study is the role of canonical (Smad) and non-canonical (Rho) signaling pathways at mediating lipid autocoid protection against TGF-β2 mediated changes in target gene expression. To determine whether mitochondrial ROS are required for canonical Smad-dependent transcriptional activity, human TM cells will be reverse transfected with 50 ng of luc2P/SBE/Hygro plasmid (a pGL4.48 6052 bp Smad Binding Element-luciferase 2P construct) using lipofectamine 20000 transfection reagent according to the manufacturer’s instructions. Transfected cells will be challenged with TGF-β2 in the presence or absence of lipid autocoids, as proposed above. ROS-targeted non-canonical signaling pathways will similarly be evaluated, including MAPK pathways ERK1/2 and p38 or the Akt pathway.

**One-year Timeline:**

**First Quarter:** Determine dose- and time-dependent effects of lipoxins and resolvins on endogenous TGF-β2 active and total protein expression and release as quantified by ELISA.

**Second Quarter:** Determine dose- and time-dependent effects of lipoxins and resolvins on endogenous TGF-β2 mRNA expression as quantified by RT-qPCR. Prepare and submit mid-year progress report.

**Third Quarter:** Determine dose- and time-dependent effects of lipoxins and resolvins on TGF-β2 signaling as quantified by immunoblot and by immunocytochemistry using commercially available antibodies. Prepare and present experimental findings at ARVO 2021.

**Final Quarter:** Prepare and submit manuscript for peer-review.

**Cited References**


IACUC Approval Letter
This *in vitro* cell culture study does not involve the use of research animals.

IRB Approval Letter
This *in vitro* cell culture study does not involve the use of living humans. Human tissue used in the preparation of primary TM cells is procured from discarded corneoscleral remnants that have been approved for use in research.