Autophagy regulation in Cyclosporine A treated lens epithelial cells

THESIS

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Abstract

Posterior capsule opacification (PCO) is the most common complication of cataract extraction. With the worldwide population aging and cataract prevalence increasing, developing novel strategies to prevent PCO is warranted. We have previously demonstrated that treating lens epithelial cells (LEC) with Cyclosporine A (CsA) can induce dysregulation of autophagy and reduce LEC viability. We established that *ex vivo* PCO could be prevented in a dose and time-dependent manner following CsA treatment. The purpose of this research was to clarify the mechanism of action by which CsA modulates regulation of autophagy in LEC. Inhibition of mTOR potently upregulates autophagy, even in the presence of sufficient nutrients and growth factors. Using cultured canine LEC, CsA-treatment decreased p-mTOR expression in a dose-dependent response. At the top of the mTOR cascade and acting as main proteins involved in autophagy initiation are protein kinase B (AKT) and adenosine monophosphate-activated protein kinase (AMPK). *In vitro* CsA-treatment did not change the intracellular AMP/ATP ratio indicating that CsA has a limited effect on the cellular energy status in LEC and may not activate AMPK. As autophagy can also be induced through CaMKKβ-mediated activation of AMPK, subsequently leading to inhibition of mTOR, a selective CaMKKβ inhibitor, STO-609, was used in conjunction with CsA treatment; autophagy was not prevented. Compound C, a specific AMPK inhibitor, did not rescue LEC from CsA-induced dysregulation of autophagy providing further confirmation that AMPK is not

involved in mTOR inhibition. By comparison, following CsA-treatment, expression of pAKT was decreased indicating that this is the more likely upstream mTOR regulator. Endoplasmic reticulum (ER) stress and accumulation of ubiquinated proteins has been previously linked to impaired autophagic flux. In canine LEC treated with CsA, accumulation of ubiquitinated proteins was observed; co-treatment with CsA and 3MA, an autophagy inhibitor, reduced ubiquitin expression. Additionally, LEC treated with both CsA and salubrinal, an inhibitor of ER-stress, decreased ubiquinated protein accumulation and increased LEC viability. This data supports the hypothesis that CsA-induces autophagy using the AKT-mTOR pathway and prolonged CsA exposure results in impaired autophagic flux.

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CHAPTER 1: INTRODUCTION AND LITERATURE REIVEW

The lens of the eye plays a vital role in vision by supporting the refraction of light on its path to the retina. In order to achieve this goal, the lens must maintain a specific structure to ensure the proper clarity and refractive nature for clear vision. The lens is a complex structure of protein packed cells that originate from the lens placode during embryological development. The lens begins to form once the neural ectoderm of the optic vesicle secretes signaling molecules to the surface ectoderm overlaying the area. The surface ectoderm then begins to thicken forming the lens placode around day 27 of gestation [1, 2]. These cells are made from placodal progenitor cells which arise from the pre-placodal region of ectoderm on the anterior neural plate [3].

The lens vesicle is initially composed of a monolayer of lens epithelial cells (LEC) lining the internal aspect of the vesicle and an external basement membrane or lens capsule. Once the lens vesicle is formed the lens will produce lens fibers in a specific orientation to promote refraction of light. These cells begin to express crystalline, which is the main lens protein responsible for the clarity and refractive nature of the lens. The primary lens fibers are evolved from the posterior LEC resulting in the embryonic nucleus; these are the only fibers formed by the posterior epithelium [2]. Subsequent to primary fiber formation, anterior LEC continually evolve into secondary lens fibers throughout life [2]. The secondary lens fibers produced *in utero* are termed the fetal nucleus. Following birth, the fetal nucleus is established and the adult nucleus and cortex are produced through lens fiber differentiation of the anterior LEC

The anterior LEC are categorized based on their metabolic and mitotic activity and role in differentiation. The central LEC have high metabolic activity in terms of ion transport and flow, but demonstrate negligible proliferation and do not contribute to fiber differentiation [4]. Mitosis of LEC occurs in the germinative zone just anterior to the lens equator. Cells produced during mitosis are directed posteriorly toward the equator and are subsequently signaled to begin the process of terminal differentiation[4]. Terminal differentiation results in loss of organelles and cellular elongation [4]. Mature lens fibers have lost all membrane-bound organelles and accumulate high cytoplasmic protein concentrations. This lens fiber network is surrounded by a lens capsule in order to help maintain the lens structure.

The lens fibers are oriented in a highly specific way to allow for light to pass through the lens with minimal scatter. The index of refraction is highest in the embryonic nucleus, at 1.406, and decreases in the external lens fibers to allow for a gradient nature in the lens's refractive capabilities [5]. To promote continued transparency throughout life, ion and nutrient flow moves through the anterior LEC into the internal lens fibers[4]. The lens fibers are connected to one another through gap junctions to facilitate transport into the deeper fiber mass [4]. As the lens continues to grow throughout life, nutrient transport becomes less effective leading to clinically evident changes in the aged lens [4].

As a human lens ages, the lens fibers become less efficient in their ability to transport nutrients and excrete waste via their junctional complexes [4]. When the lens becomes unable to maintain clarity due to this physiological efficiency deficit, the lens begins to opacifiy. Eventually this loss of clarity becomes symptomatic to patients who

typically present with night vision difficulties that can advance to overall decreases in visual acuity. When these metabolic lens changes are observed on clinical evaluation with associated patient symptoms, a diagnosis of cataracts is made. Cataract is defined by the American Optometric Association as “a cloudy or opaque area in a clear lens.” Cataract onset typically occurs in the sixth decade; however, onset can vary based on a number of genetic and epidemiological factors. Cataracts cause a significant loss of quality of life making them an important age-related vision diagnosis around the world. Cataracts account for 51% of global blindness making them the leading cause of blindness worldwide [6]. The prevalence of cataract-related blindness is expected to reach 30.1 million by 2020 in the United States alone, making treatment of cataract an important priority for healthcare [7].

There are several types of cataract including nuclear sclerotic, cortical, and posterior subscapular cataracts. Certain systemic and ocular diseases can also cause specific types of cataracts. Cataract can only be treated with surgical extraction through a surgical procedure known as extracapsular cataract extraction (ECCE). Current standards of cataract surgery start with the creation of a primary incision and paracentesis into the limbal region of the cornea by a scleral tunnel incision, clear cornea incision, or femtosecond laser [8]. Following creation of the incision, a capsulorhexis is created by removing the central anterior capsule of the lens. Phacoemulsification, an ultrasonic device with suction, is then used to remove the opacified lens fibers out of the lens capsule [8]. Following removal of the cataract, the lens capsule is polished and typically, the lens fibers are replaced with a silicone or acrylic intraocular lens (IOL) placed within

the lens capsule. The IOL uses the capsule as support to remain in place. Patients are typically placed on post-operative drops consisting of a steroid, topical NSAID, and antibiotic.

As with any surgical procedure, cataract surgery can have a variety of complications, such as a retinal detachment or endophthalmitis. Because cataract surgery is such a common surgical procedure, limiting these complications is essential in reducing healthcare costs and obtaining successful patient outcomes. The most common side effect of cataract surgery is posterior capsular opacification (PCO) or secondary cataract [9]. The onset of PCO ranges from months to years after ECCE and occurs when residual LEC migrate and proliferate within the lens capsule causing fibrosis [10]. The prevalence of PCO following ECCE is approximately 38.5% three years post operatively [11]. Regardless of the three year prevalence the lifetime incidence of PCO is up to 50% [8]. Additionally, there has been a significant amount of research demonstrating that pediatric patients are at a higher risk for developing PCO [12].

An Nd: YAG Capsulotomy is currently the only treatment for PCO. The Nd: YAG laser removes the fibrotic posterior capsule directly behind the IOL in the central visual axis in order to restore clear vision. Although Nd: YAG Capsulotomy is relatively safe, the procedure can cause hyphema, intraocular pressure spikes, cystoid macular edema, and many other complications [8]. As such, developing novel strategies to prevent PCO is important.

Dozens of chemical compounds targeting the LEC have been analyzed to determine their role in preventing such a visually devastating side effect. Chemical

compounds classified as simple osmotic effective solutions to cytostatic agents have been researched in order to find an effective agent to use pre-, peri-, or post- surgery in order to limit LEC re-growth [13]. To date, none of these compounds have been found to effectively inhibit *in vivo* PCO formation. Research on IOL design and material has been shown to alter the risk of developing PCO. Foldable IOLs and sharp haptic edges have been shown to reduce or slow PCO progression [14]. According to a Cochrane review on PCO prevention, IOL material does not play a significant role preventing prevalence of PCO; however, silicone IOLs tend to develop more severe PCO than acrylic [15]. Use of postoperative anti-inflammatory medications have also been evaluated but have not been found to significantly change the prevalence of PCO [15]. Modifying surgical methods, such as extensively polishing the lens capsule, have been tested in order to see if PCO can be prevented. While there is limited data suggesting modified surgical methods can reduce long-term PCO progression, no specific cataract surgery technique can prevent the complication [16]. As such, PCO persists as a worldwide problem and a method to prevent PCO in its entirety has yet to be developed.

Cyclosporine (CsA) is an immunosuppressive agent that inhibits calcineurin resulting in decreased levels of interleukin 2 (IL-2), preventing T-Cell replication [17]. Cyclosporine has been proven to be a successful treatment for inflammatory dry eye, as well as in dermatological conditions and for decreasing transplant rejection [18, 19] . Cyclosporine treatment in human LEC *in vitro,* has previously been shown to inhibit cell growth; however, the mechanism by which proliferation was decreased was not previously determined [20]. Other data has shown that using CsA during cataract

extraction can reduce the incidence of PCO postoperatively. One study showed success using microspheres made of polylactic acid, polyglycollic acid, and 20% CsA injected into the capsule of rabbit eyes following ECCE. Significant PCO was observed in 35% of the CsA-treated rabbits, but clinically less severe than the control rabbits and with a later onset. This experiment also showed little to no anterior chamber reaction, IOP spikes, or signs of corneal edema in response to CsA [21]. Another study using the implanted IOL itself as the delivery device, demonstrated a decrease the presence and severity of PCO with little to no side effects to the rest of the rabbit eye [22]. More recent evidence indicates that treating LEC with CsA results in a dose-dependent induction of autophagy with a subsequent reduction in cellular viability [23].

Autophagy is Greek for "eating of self" and was coined by Christian de Duve when he discovered mitochondrial cell death with increases in glucagon [24]. Autophagy is mediated through lysozymal encapsulation of intracellular structures, such as organelles or malfunctioning proteins [25]. This is a normal process thought to maintain cellular viability, particularly in times of cellular stress. Dysregulation of autophagy can promote cell death and has been described in research analyzing pathways involved in cell overgrowth [25]. The mechanistic target of rapamycin complex (mTOR) pathway has been a leading pathway in autophagy research; inhibition of mTOR induces autophagy. mTOR is a protein kinase that when in complex regulates diverse cellular functions. mTOR is found in two major complexes, mTORC1 and mTORC2. mTORC1 is made up of the mTOR serine/theorine protein kinase, regulatory associated protein of mTOR (RAPTOR), and four associated proteins that activate or inhibit the complex [26].

mTORC1 functions include protein synthesis and autophagy regulation [27]. By comparison, mTORC2 plays a role in metabolism and cell structure and is thought to play a lesser role in autophagy [27]. mTOR malfunction has shown importance in Type II diabetes, cancer, and neurological disease [27].

A number of factors (**Figure 1**) can influence the activity of mTORC1 including amino acid levels, stress, oxygen, and growth factors [27]. These external stimuli can activate protein kinase B (AKT) resulting in increased mTORC1 activity through two main mechanisms. One mechanism is through the phosphorylation of the downstream protein proline rich AKT substrate (PRAS40), which blocks the ability of PRAS40 to inhibit mTORC1 on the RAPTOR subunit, resulting in mTORC1 activity [28]. An alternate mechanism of inducing mTOR activity via the AKT pathway is through the tuberin (TSC1/TSC2) complex. Activated AKT phosphorylates TSC2, inhibiting its ability to eliminate suspected mTOR substrates. By preventing TSC2 from hydrolyzing RHEB-GTP , mTOR activity increases by increasing RAPTOR activation [29]. There is evidence that with significant mTORC1 activity a negative feedback loop will downregulate AKT expression in the pathway.

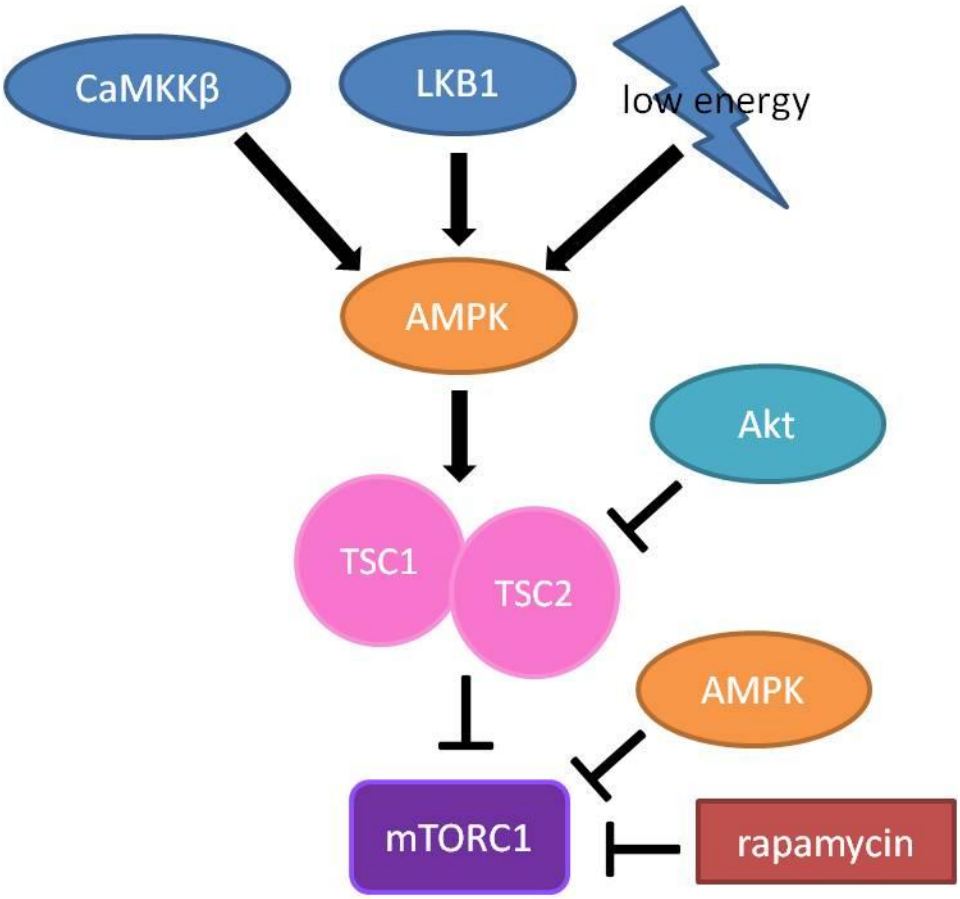

Figure 1. Regulation of mTOR. Low energy, LKB1, and CaMKKβ can activate AMPK, resulting in activation of the TSC1/TSC2 complex, with subsequent inhibition of mTORC1. Alternatively, the TSC1/TSC2 complex can be inhibited by AKT resulting in mTORC1 activation. Additional proteins can regulate the pathway; mTORC1 can be directly inhibited via rapamycin, AMPK, and TSC1/TSC2.

The TSC1/TSC2 complex can also be regulated by Adenosine Monophosphate Kinase (AMPK), which is controlled by cellular levels of AMP and ATP. Adenosine Monophosphate Kinase is made up of three subunits that allosterically bind together

causing activation of the complex. Elevated levels of AMP in a cell indicate low levels of energy in the cell. When AMP binds to the alpha subunit of AMPK, the kinase can then directly inhibit mTORC1 by binding RAPTOR or indirectly inhibit mTORC1 via TSC2 [30]. During indirect inhibition of mTORC1, TSC2 creates a heterodimer with TSC1 that hydrolyzes RHEB-GTP to RHEB GDP reducing available GTP, a known mTORC1 substrate [30]. Although AMPK is an energy sensor for AMP and ATP, other proteins can control AMPK activity such as phosphorylation by LKB1 tumor suppressor kinase or high levels of intracellular calcium via Ca2+/calmodulin-dependent protein kinase kinase-β (CaMKK-β) [31].

mTORC1 plays a role in autophagy by keeping autophagy-inducing proteins inactive. In other words, when mTORC1 is inactive, the lack of phosphorylated control downstream results in the induction of autophagy. Specifically, mTORC1 is suspected of controlling the ULK1/Atg13/FIP200 protein complex, which plays a role in initiating autophagy [32]. Through phosphorylation, Atg13 is inhibited by mTORC1 when the enzyme complex is active in order to prevent autophagy [32]. Besides mTORC1 inhibition directly inducing autophagy, there are also reports that AMPK can directly interact with Ulk1 assisting in autophagy [33].

Beyond the mTORC1 pathway, the endoplasmic reticulum (ER) has been shown to play a role in autophagy as well. When fibroblasts were treated with drugs proven to induce ER stress, cells exhibited signs and biological markers of autophagy, with concurrent downregulation of AKT/mTORC1 activity (**Figure 1)** [34]. This concurrent downregulation provides a functional linkage between mTORC1 signaling cascade and

ER stress. There is also research supporting the hypothesis of a feedback loop between mTOR expression and induction of ER stress, such that activation of the mTOR pathway results in a reduction of ER stress markers [34]. One proven autophagy marker is the upregulation of the LC3-phosphatidylethanolamine conjugate (LC3-II), which is a microtubule associated protein involved in autophagosomal membrane formation [35]. LC3-II expression correlates with the number of autophagosomes present within a cell and is increased following CsA treatment [23]. Induction of ER stress can result in increased LC3-II expression and dysregulation of autophagy may contribute to ER stress-induced cell death [36-38]. In support of ER stress-induced autophagy, cells treated with the well-known autophagy inhibitor 3-methyladenine (3-MA), demonstrate improved cell viability and a decrease in ER stress markers [39].

Another marker of autophagy is polyubiquitin-binding protein p62/SQSTM1 (p62), which has been found to have a multidimensional role in autophagy [40]. The signaling adapter p62 protein is a specific target for autophagic degeneration and has been shown to contribute to the formation of ubiquinated protein aggregates. p62 mediates delivery of these aggregates to the autophagy system, serving as a readout of autophagic degradation. Additionally, p62 can accumulate within a cell and can be considered a marker of autophagic flux inhibition [40]. Within the ER itself, proteins that are misfolded and targeted for degradation are tagged with ubiquitin. As such, increased ubiquitin can be used as a marker for cell stress [37].

The purpose of this research is to clarify the mechanism of action by which CsA induces autophagy in LEC. Specifically, the importance of AKT and AMPK in the

regulation of autophagy in the lens will be determined through use of chemical and biologic inhibitors. Furthermore, this research will begin to address the role of ER stress in CsA-induced autophagy and loss of cell viability. The importance of this work is to identify the molecular mechanisms by which CsA could target LEC viability in order potentially prevent PCO.

CHAPTER 2: MATERIALS AND METHODS

Cells and Tissues

Normal canine LEC were obtained from animals, in overall good health, that were euthanized for an unspecified and unrelated reason. All protocols are in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and have been approved by The Ohio State University Institutional Animal Care and Use Committee.

Globes were enucleated within 1 hour of euthanasia, rinsed in 10% povidone-iodine solution, and stored in phosphate buffered saline (PBS) at 4^{o}C for up to 12 hours prior to tissue dissection. To obtain the lens capsule, the cornea was first removed by making a groove 1 mm posterior to the limbus, followed by a stab incision with a scalpel blade into the anterior chamber. The cornea was removed by cutting along the groove with Steven's tenotomy scissors taking care to point the scissors tips away from the lens to prevent any damage to the capsule. Next, Colibri forceps and Vannas scissors were used to remove the iris from the ciliary body. Following removal of the iris, the lens capsule was obtained via capsulorhexis; a 26 g needle was used to make a small incision and Utrada forceps removed the axial anterior capsule.

The capsule was then incubated with trypsin-EDTA (0.25% Gibco, Carlsbad, CA) at 37^{o}C for 5 minutes. The solution and lens capsule were centrifuged at 1800 rpm for 5 minutes. The supernatant was decanted and the cell pellet was resuspended in

Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 1% antibiotic/antimycotic (Gibco) and 10% fetal bovine serum (FBS; Gibco). The solution and cells were places into a laminin coated flask (Beckton-Dickinson, Franklin Lakes, NJ) and incubated in a humidified incubator at 37^{o}C and 5% CO_2. Cells were fed every 2 – 3 days and were grown to approximately 90% confluence prior to re-plating.

Treatment of Primary Cell Cultures

For investigation of cell-signaling pathways involved in LEC autophagy, cultured canine LEC were treated with various compounds (**Table 1**) in combination with cyclosporine A (CsA; LC Laboratories, Woburn, MA). The effects of the compounds were investigated by Western blot analysis and immunofluorescence. For each compound investigated, LEC were passed into 12-well laminin coated plates (Beckton-Dickinson). All final treatment preparations that were applied to the canine LEC were diluted in incomplete Modified Eagle Medium (MEM; Gibco) containing 1% antibiotic/antimycotic. Photomicrographs of the cells were taken at time points 0, 4, 8, 12, 24, 36, and 48 hours. Any morphologic changes in each treatment group were noted at each observation.

Compound	Function	Manufacturer Location	Concentration	Application
CsA	Induce autophagy	LC Laboratories	20 µM	Western blot, immunofluorescence
Rapamycin	Induce autophagy (positive control)	LC Laboratories	10 µM	Western blot
STO-609	CaMKK-β inhibitor	Santa Cruz Biotechnology (Dallas, TX)	10 µM	Immunofluorescence
Compound C	AMPK direct inhibitor	Millipore Sigma (Burlington, MA)	10 µM	Immunofluorescence
Salubrinal	Inhibits ER Stress	Santa Cruz Biotechnology	10 µM	Western blot

Table 1: Compounds used for investigation of cell signaling pathways in CsA-treated LEC.

Immunofluorescence Staining

Canine LEC, prepared as above, were rinsed twice with room temperature PBS for five minutes per rinse, carefully aspirating fluid as to not disrupt the cells on the cover slip. Cells were then fixed with a 3% paraformaldehyde suspension for twenty minutes, followed by two more rinses, 5 minutes each, with PBS. Canine LEC were permeabilized with PBS containing 0.1% Tween 20 (PBS-T) for 10 minutes, followed by two, five minute washes of PBS-T. Blocking buffer made of PBS-T and 10% goat serum (Vector Laboratories, Burlington, CA) was then applied to each well for one hour at room temperature. Following the blocking, a solution of primary antibody with background reducing agent (DAKO Agilent, Santa Clara, CA) was applied to the cells. Multiple primary antibodies were used during these experiments to label multiple targets, as listed in Table 2. The primary antibody was applied to the cells for one hour at 37°C. Following treatment with the primary antibody, the cells were rinsed with PBS-T three times for 5

minutes each. Secondary antibody was applied for one hour at room temperature. Following incubation with the secondary antibody, the cells were washed three times for 5 minutes with PBS-T. DAPI nuclear stain (Molecular Probes, Waltham, MA) was prepared at a concentration of 300 nm in PBS and placed onto the cells following the last rinse of PBS-T for 5 minutes. Three more 5 minute rinses of PBS followed the DAPI treatment. The slips were then mounted on a slide with Prolong Gold antifade reagent (Molecular Probes) and a cover slip. Clear coat nail polish was then applied to edges of the slip to prevent drying and preserve the sample.

Primary Antibody	**Manufacturer, Location**	**Dilution**	**Secondary Antibody**	**Application**
LC3	Novus Biologicals, (Littleton, CO)	1:200	Goat anti-rabbit IgG-AlexaFluor488 (Thermo Scientific)	Immunofluorescence
Phospho-mTOR	Cell Signaling (Danvers, MA)	1:1000	Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology)	Western blot
LKB1	Cell Signaling	1:1000	Goat anti-rabbit IgG-HRP	Western blot
pAKT	Cell Signaling	1:200		Western blot
Ubiquitin	Santa Cruz Biotechnology	1:200	Goat anti-mouse IgG-HRP (Thermo Scientific)	Western blot
β-actin	Thermo Scientific (Waltham, MA)	1:10,000	Goat anti-mouse IgG-HRP	Western blot

Table 2: Antibodies used throughout the study.

Western Blot of Lens Epithelial Cells

Whole cell protein was extracted from treated LEC using ice cold lysis buffer. The lysis buffer contained 150 mM sodium chloride, 1.0% Triton X-100, and 50 mM Tris, pH 8.0. Once each well was covered with lysis buffer, the plate was placed on an agitator for 30 minutes at 4^{o}C.A cell scraper was used to scrap the bottom of the wells and the mixture was then placed into microcentrifuge tubes and centrifuged (12,000 rmp) at 4^{o}C for 20 minutes. Acetone was used to precipitate the protein in which four-times the volume of the supernatant of acetone was added to the tubes. The tubes were then gently vortexed before the solution was allowed to rest at -20^{o}C for 48 hours. The solution was then centrifuged at 4^{o}C for 10 minutes at 13,000 rpm. The subsequent supernatant was discarded without disturbing the protein pellet. The pellet was suspended in water and the protein concentration was determined using the Pierce 660nm Protein Assay (Thermo Scientific). The protein samples were denatured for 3 minutes at 95^{o}C in SDS sample loading buffer with 5% β-mercaptoethanol (Thermo Scientific), and 15 µg of protein was separated by SDS-PAGE (10% acrylamide, v/v) at 125 V for approximately 3 hours. Following the electrophoresis the semi-dry transfer process onto nitrocellulose membrane was run at 300 milliamps for 1 hour.

Blocking Solution (1x TBS-T with 5% dry milk) was placed over the blot and was allowed to gently rock at 4^{o}C overnight. Primary and secondary antibody incubations were performed in blocking solution; antibodies used for immunoblotting are outlined in Table 2. Primary antibodies were incubated at room temperature for three hours or at 4^{o}C overnight, membranes were washed in TBS-T, and secondary antibodies were incubated for 1 hour at room temperature. The blot was again rinsed and protein was detected using

chemiluminescent substrate for horseradish peroxidase (HRP; SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific). The blot was imaged and densitometry reading obtained with a Kodak Image Station 4000MM (New Haven, CT). Antibodies were stripped from the membranes (Thermo Scientific) and immunoblotting was repeated using anti-β-actin antibody for confirmation of equal protein concentrations in all lanes.

ATP Assay

An ATP Assay Kit (Abcam, Cambridge, UK) was used to evaluate the AMP: ATP ratio following the manufacturer's recommendations. Briefly, LEC were passed into a 6-well laminin-coated tissue culture plates and grown to 90% confluence. Cells were treated with vehicle, 5 μg/mL CsA, or 10 μg/mL CsA for 24 or 48 hours. Following treatment, cells were resuspended in ATP Assay Buffer and homogenized. Samples were centrifuged for 5 minutes at 4^{o}C at 13,000 *g* to remove any insoluble materials. To remove enzymes that can interfere with the assay, samples underwent deproteinization by adding 4 M perchloric acid to each sample. Samples were then incubated on ice for 5 minutes and centrifuged at maximum speed for 2 minutes. To precipitate the perchloric acid, an equal volume of 2 M potassium hydroxide was added to each sample. Samples were centrifuged at 13,000 x*g* for 15 minutes at 4^{o}C and subsequently kept on ice. ATP reaction mixture was then added to each sample and allowed to incubate at room temperature for 30 minutes. Cellular ATP concentrations of the samples and standards were quantified using a microplate (Tecan Infinite M200 Microplate Reader; San Jose, CA) at OD 570 nm.

Statistical Analysis

The analysis of variance (ANOVA; SPSS statistical software) was used to assess the treatment effects on protein expression and AMP/ATP levels. The Tukey's post-hoc test was used to determine the significance between treatment groups. A p-value that was less than or equal to 0.05 was considered to be significant.

CHAPTER 3: RESULTS

Effects of CsA Treatment on mTOR expression in LEC

It has previously been demonstrated that 5 to 15 µg/mL CsA will induce LEC autophagy [23]. To further characterize CsA-induced autophagy, expression of mTOR was first evaluated. As expected, LEC treated with CsA for 48 hours underwent morphologic changes consistent with induction of autophagy, such as vacuole formation (**Figure 2**). Rapamycin is a known inducer of autophagy and was used as a positive control (**Figure 2**). Western blot analysis of phosphorylated (p-) mTOR revealed decreased expression following CsA treatment in a concentration dependent manner; p-mTOR was also reduced in rapamycin treated LEC (**Figure 3**).

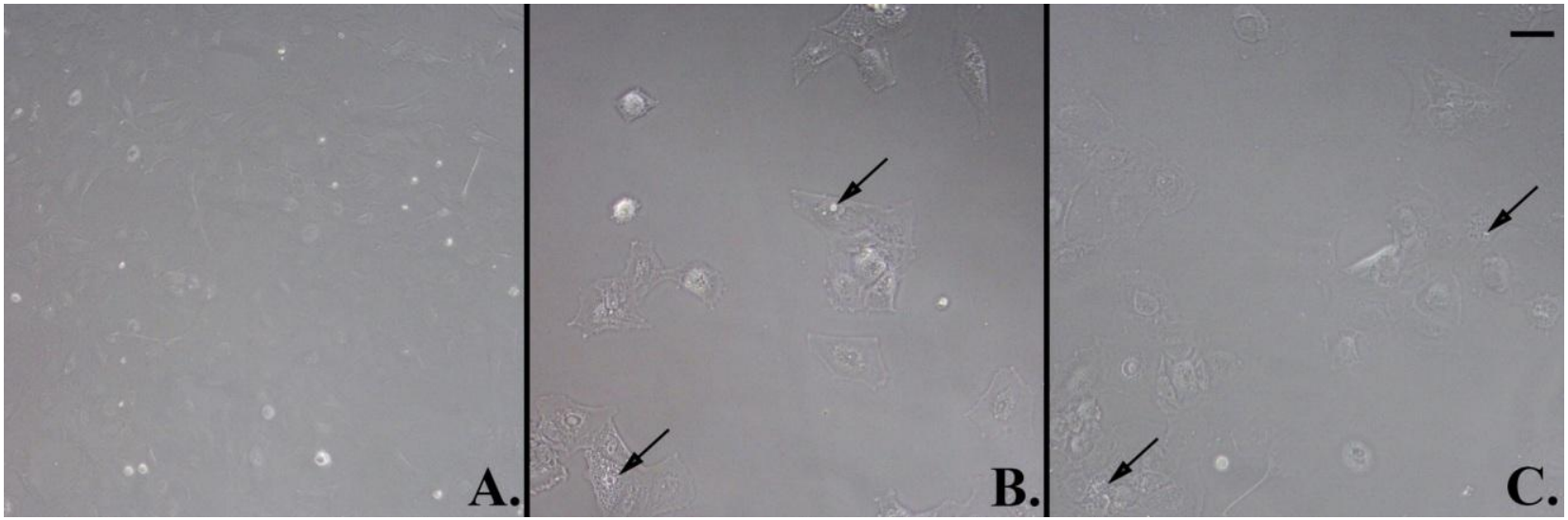

Figure 2. Morphologic changes in treated canine LEC. Using light microscopy **(A)** vehicle-treated LEC maintained cellular confluence, adherence to the underlying culture dish, and normal morphology. By comparison, LEC treated with **(B)** 10 µg/mL CsA or **(C)** 10 µM rapamycin had prominent vacuole formation (arrows) and reduced cell density. Scale bar is equal to 250 µm.

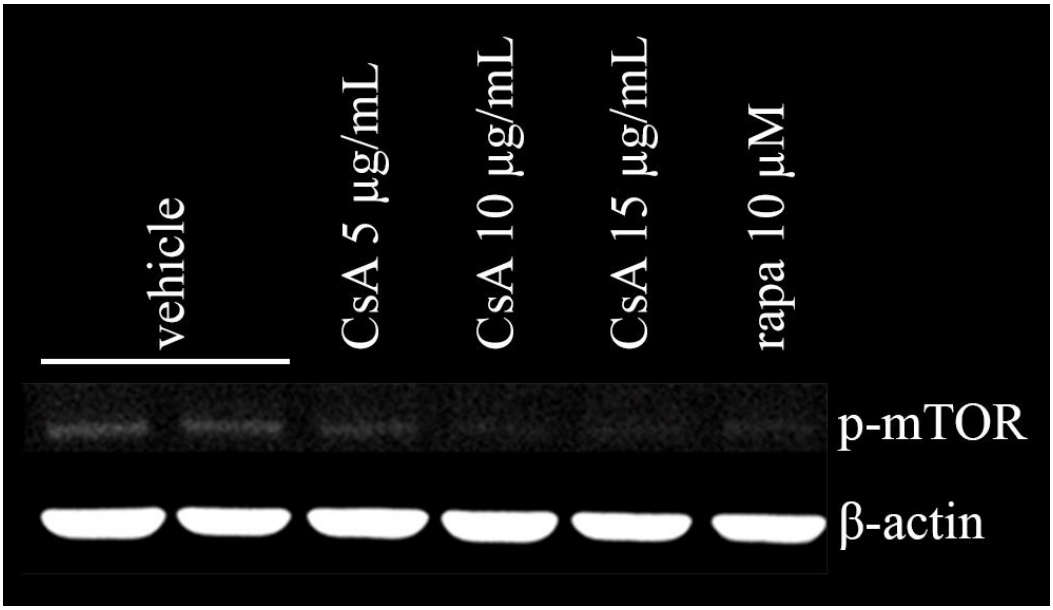

Figure 3. p-mTOR expression in treated canine LEC. Evaluation of activated mTOR by western blot demonstrated reduced expression with increased concentrations of CsA. Rapamycin was used to confirm inhibition of p-mTOR. β-actin as a loading control for each sample.

Characterizing the regulation of CsA-induced mTOR inhibition

As seen in **Figure 1**, mTOR can be regulated by AMPK and Akt. While AMPK can directly modulate mTOR expression, AMPK itself can be regulated by changes in the cellular energy status, by LKB1, or by CaMKKβ [30, 31]. The first experiment evaluated the cellular energy status in CsA-treated LEC to determine if a decrease in available ATP was responsible for inhibiting AMPK. The AMP/ATP ratio remained stable throughout the treatment course (**Figure 4**).

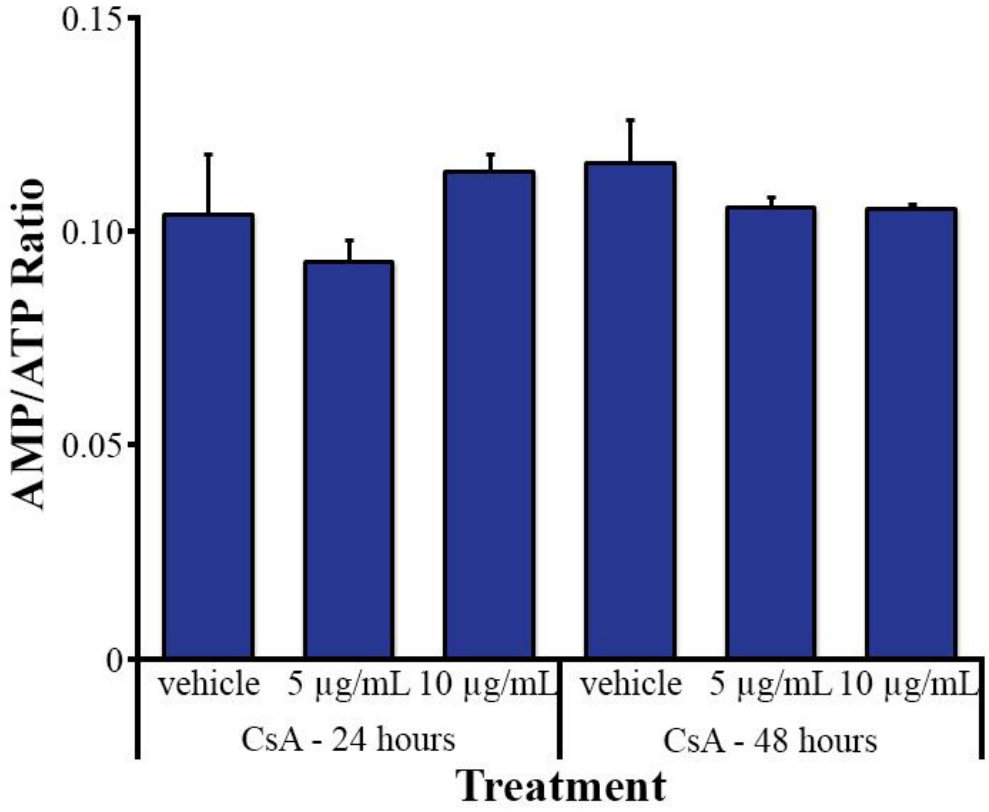

Figure 4. Ratio of AMP/ATP in canine LEC following CsA treatment. AMP/ATP levels in treated LEC remained unchanged with varying concentrations of CsA over time.

To determine if CsA increased LKB1, thus potentially activating AMPK to induce autophagy (**Figure 1**), western blot analysis was used. As observed in **Figure 5**, LKB1 levels did not significantly change between treatment groups

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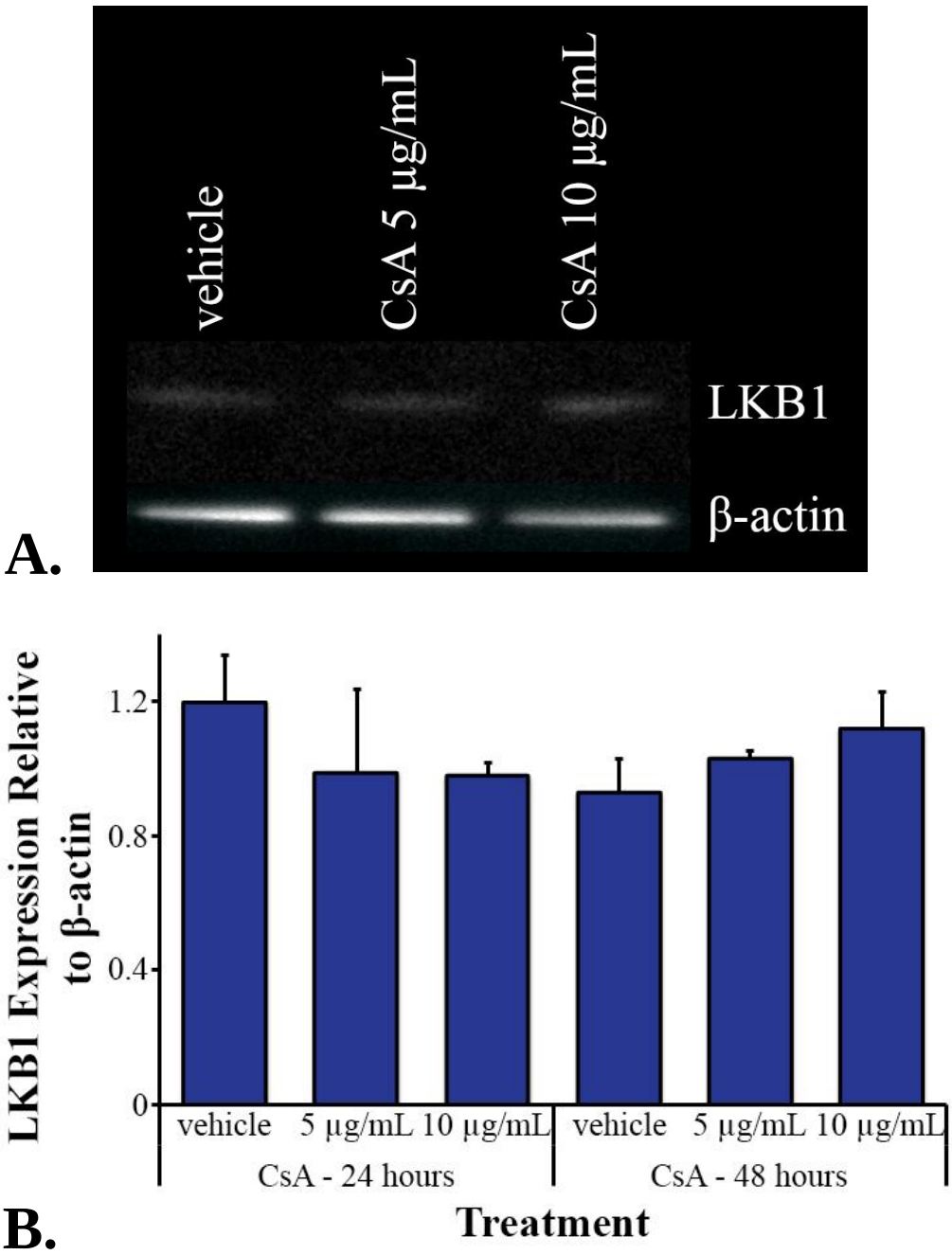

Figure 5. mTOR regulation is independent of LKB1. Evaluation of LKB1 expression with (**A**) western blot analysis demonstrated no change following treatment with either 5 or 10 µg/mL CsA. β-actin was used as a loading control. (**B**) Quantification of LKB1 expression relative to β-actin.

To evaluate the role of CaMKK-β in AMPK and p-mTOR regulation, STO-609 was used to block CaMKK-β. **Figure 6** shows visible CsA-induced vacuole formation and cell loss in cultured LEC compared to cells treated with vehicle or STO-609 alone. When LEC were co-cultured with CsA and STO-609, inhibition of CaMKK-β did not rescue cells from CsA-induced morphologic changes. To further clarify if STO-609 could prevent or reduce CsA-induced autophagy, the number of LC3-II-positive cells were calculated; no significant changes in LC3-II expression was observed when comparing cells treated with only CsA to those treated with CsA and STO-609 (**Figure 6**).

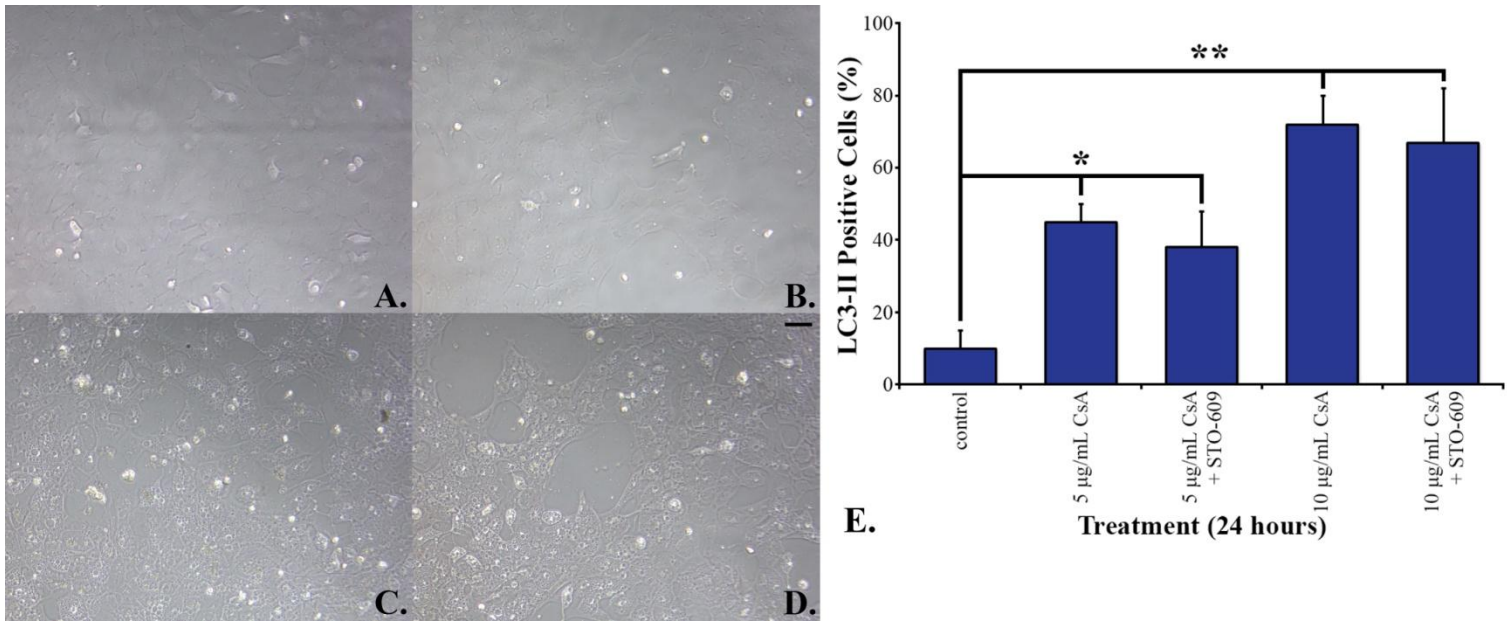

Figure 6. CaMKK-β inhibition does not prevent CsA-induced autophagy. Canine LEC treated with (**A**) vehicle or (**B**) STO-609 alone demonstrates normal morphology and cellular density. As expected, treatment with (**C**) 10 µg/mL CsA alone induced vacuole formation and decreased cellular viability. Cells treated with (**D**) CsA (10 µg/mL) and STO-609 also demonstrate reduced viability and vacuole formation. Scale bar is equal to 250 µm. Following immunofluorescent labeling, (**E**) CsA, with or without STO-609, significantly increased the number of LC3-II positive cells compared to control (* $p<0.01$; **$p<001$). The number of LC3-II positive LEC was not significantly altered when comparing CsA treated to CsA and STO-609 treated.

Finally, to determine if AMPK itself was involved in CsA-induced autophagy, LC3-II expression was evaluated in the presence or absence of Compound C. Compound C is a direct AMPK inhibitor. As observed in **Figure 7**, LC3-II expression is observed equally in cells treated with CsA alone or when co-cultured with CsA and Compound C.

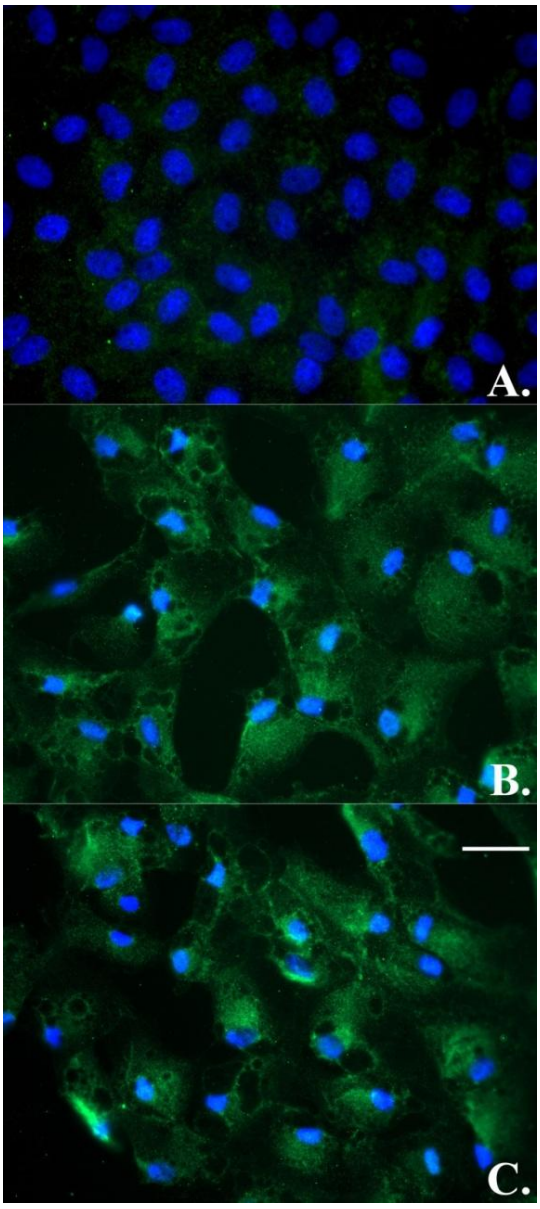

Figure 7. AMPK inhibition does not inhibit induction of autophagy. In canine LEC, LC3-II immunofluorescence is minimally observed in (**A**) cells treated with Compound C alone. By comparison, cells treated with (**B**) with CsA (10 µg/mL) have increased LC3-II expression; this is similar to LC3-II expression in (**C**) LEC treated with CsA and Compound C. Scale bar is equal to 50 µm.

Since AKT is a known regulator of mTOR, it was necessary to evaluate AKT expression when LEC were treated with CsA. Western blot analysis determined significantly reduced p-AKT expression with increasing concentrations of CsA over a 24 hour treatment period (**Figure 8**).

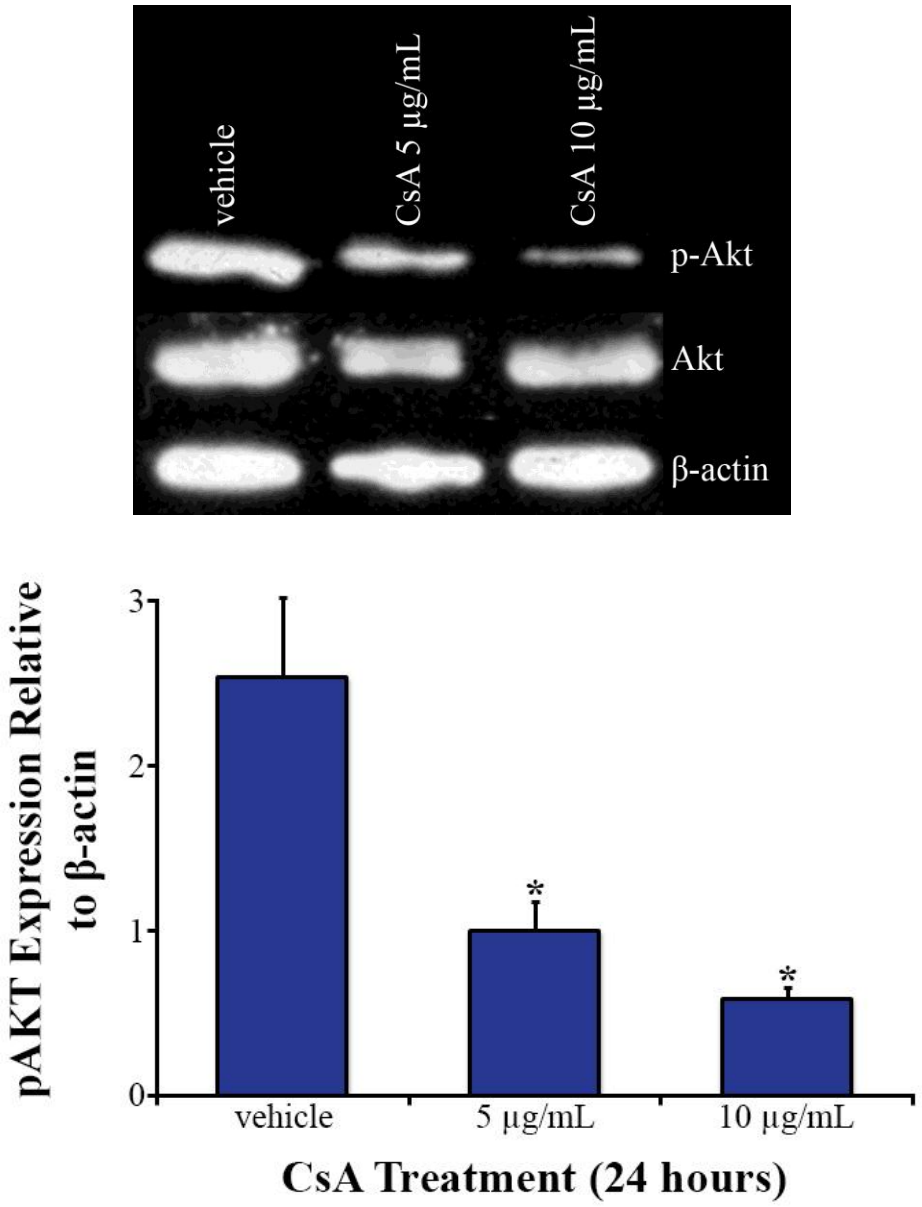

Figure 8. mTOR regulation involves activation of AKT. With western blot analysis, (**A**) evaluation of p-AKT expression was decreased following treatment with either 5 or 10 µg/mL CsA. Total AKT expression remained unchanged with CsA treatment. β-actin was used as a loading control. (**B**) Quantification of pAKT expression relative to β-actin.

Evaluating Endoplasmic Reticulum Stress in CsA-Treated Cells

This experiment's purpose was to determine if CsA affects the ER stress pathway. Misfolded proteins within the ER stress pathway can cause the cell to have dysregulation of autophagic flux leading to loss of cell viability. Ubiquitin expression can be used as a broad estimate of misfolded proteins and thus can be used to evaluate ER stress. **Figure 9** demonstrates that when LEC are treated with CsA alone, there is an increase of ubiquitin. Because the antibody used in the current study was designed to detect all ubiquitin, any protein tagged with ubiquitin will be detected in the western blot. As such, a single protein band is not expected to be observed in the western blot, rather, multiple bands demonstrating all ubiquintinated proteins in expected. When cells are co-cultures with salubrinal, a known ER stress inhibitor, and CsA the cells maintain viability and have decreased ubiquitin expression (**Figure 9**). Cells treated with CsA and 3-MA, an autophagy inhibitor, also demonstrated ubiquitin expression similar to the vehicle treatment (**Figure 9**). As expected, cellular density decreased following CsA treatment; however, when LEC were co-cultured with CsA and salubrinal, cellular density was similar to controls (**Figure 10**)

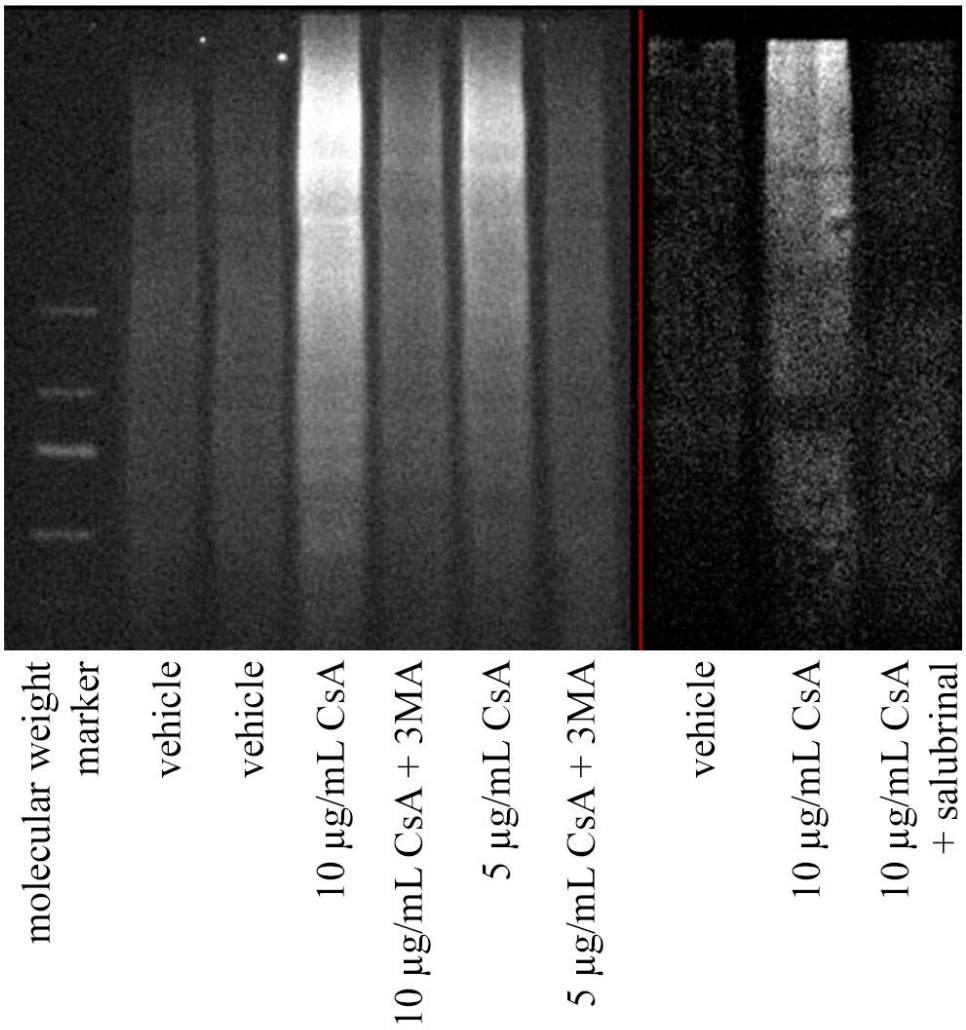

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Figure 9. CsA increases the presence of ubiquinated proteins in canine LEC. Both 5 and 10 µg/mL CsA increase ubiquitin expression, while treatment with CsA and 3-MA reduces ubiquitin expression. LEC treated with salubrinal and CsA show little expression of ubiquitin, when compared to CsA treated LEC.

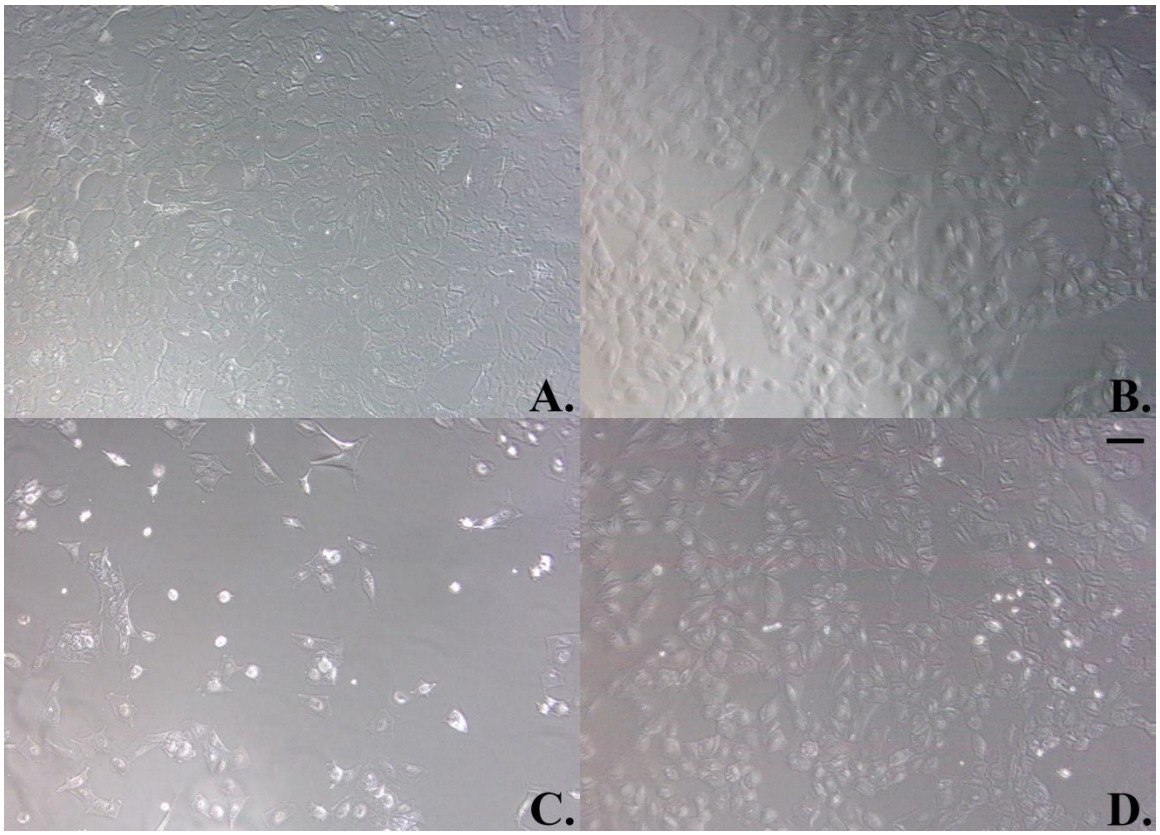

Figure 10. Inhibiting ER stress maintains cellular density with CsA treatment. Canine LEC treated with (**A**) vehicle or (**B**) salubrinal alone maintain normal morphology and density; (**C**) CsA (10 µg/mL) treatment induces vacuole formation and cell loss. When LEC are co-cultured with (**D**) CsA (10 µg/mL) and salubrinal, vacuole formation is substantially reduced and cellular density in improved. Scale bar is equal to 500 µm.

CHAPTER 4: DISCUSSION

With an aging population, cataract prevalence is expected to rise in both the United States and globally. According to the World Health Organization, cataracts are still responsible for a majority of worldwide blindness [6]. More recent data from the Global Burden of Disease Study 2015 database shows there has been an 89% increase in disability-adjusted life years between 1990 and 2010, making cataract the third most disabling disease, following anemia and hearing loss [41]. Successful removal of these cataracts with surgery can improve the quality of life of effected patients. Posterior capsular opacification is the leading complication of cataract surgery causing visually significant deficits in up to 50% of cases [8]. The only currently available treatment for PCO is a secondary procedure known as Nd: YAG capsulotomy. Preventing PCO altogether would save the healthcare industry substantial money in post-surgical care costs and lessen patient disability. In addition, with the eradication of PCO, patients receiving cataract surgery in low socioeconomic areas of the world, especially those with limited access to healthcare, would not be burdened by secondary vision loss.

With the lifetime prevalence of PCO being 50% post-cataract surgery, a non-surgical method of treatment would be advantageous in today's world [8]. Cyclosporine has been shown to induce LEC death *in vitro*, making it a potential strategy for PCO prevention [20, 23]. Cyclosporine has proven effective in decreasing the prevalence and severity of PCO in rabbits using either extended release microspheres or CsA-embedded

IOL devices [21, 22]. Pei, et al. has also demonstrated that intraocular CsA has little to no negative consequences on other ocular structures [21]. Here, we find evidence supporting that CsA induces autophagy through components of the mTOR pathway. With the understanding that CsA inhibits mTOR expression, we next sought to characterize upstream regulation of mTOR inhibition (**Figure 10**).

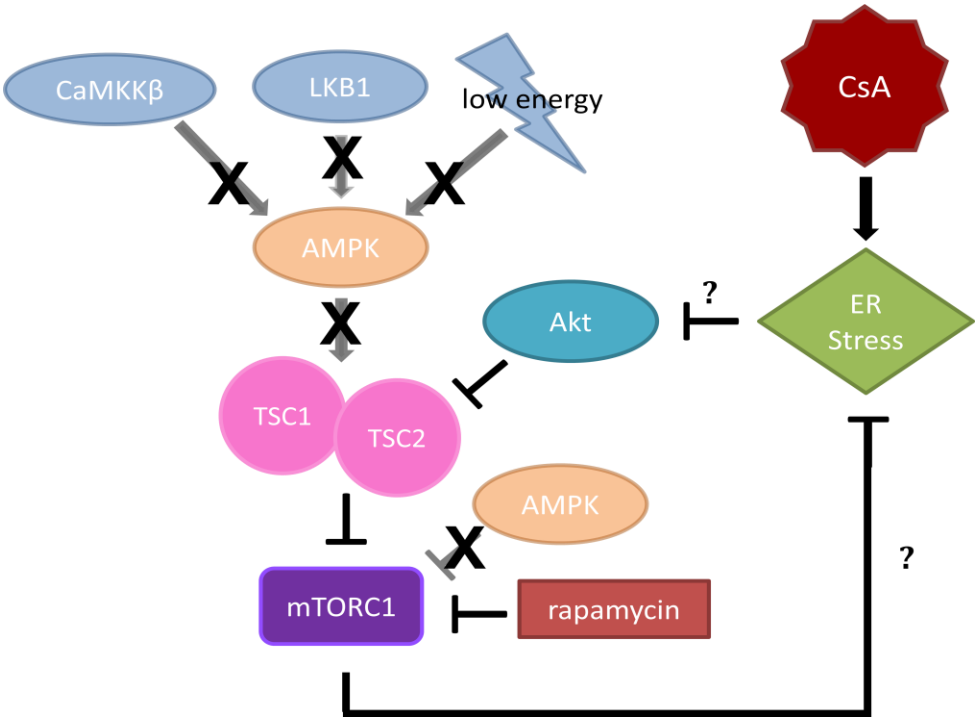

Figure 11. Proposed Model for CsA-Induced Regulation of mTOR. Following CsA treatment, there were no changes in cellular energy status or expression of LKB1. Inhibition of CaMKKβ or AMPK did not rescue LEC from CsA-induced autophagy. CsA treatment inhibited expression of pAKT and induced ER stress. This research did not define the interaction between ER stress and AKT, nor did this research elaborate on a potential feedback mechanism between autophagy and ER stress.

Adenosine Monophosphate Kinase is a major protein in the mTOR pathway and as such, its role in CsA-induced cell death was evaluated. As shown in **Figure 1**, AMPK can either directly or indirectly inhibit mTOR [42]. Activation of AMPK was not directly evaluated here due to the lack of a canine-reactive AMPK antibody. However, to indirectly determine the role of AMPK in mTOR regulation, regulators of AMPK were subsequently evaluated. Binding of AMP enhances AMPK activation while ATP oppositely regulates the activity of AMPK. This activation of AMPK under low energy conditions, results in mTOR inhibition and autophagy induction [30]. Cellular energy status was evaluated via an ATP assay since the ratio of AMP to ATP is the most accurate way to measure the intracellular energy level. Levels of ATP remained virtually unchanged, indicating that CsA did not lower the energy status of treated LEC. This suggests that CsA-induction of autophagy is not regulated by changes in the AMP/ATP ratio.

The constitutively active LKB1 is another AMPK-phosphorylating kinase that could play a role in CsA-induced autophagy. Several previous studies have shown that LKB1 is important in maintaining cellular homeostasis and regulating autophagy [43]. In rat hippocampal cells, CsA treatment led to increased phosphorylation of both LKB1 and AMPK; the study did not directly evaluate autophagy [44]. Others have also demonstrated that activation of the LKB1-AMPK pathway can lead to increased autophagy and loss of cell viability confirming with both *in vitro* and *in vivo* evidence the potential relevance of LKB1 regulation of mTOR [45, 46]. In our study, analysis of

LKB1 kinase expression in LEC revealed that LKB1 did not significantly change in response to CsA treatment (**Figure 5**). While this result differs from some previously published research, there is substantial evidence demonstrating that regulation of AMPK and/or mTOR can occur in an LKB1-independent manner. As examples, LKB1 siRNA failed to prevent autophagy in flavonol-treated hepatocytes [47]. Previously published data has also found that prostate cells treated with CsA had unaltered levels of LKB1 and rather, used CaMKK-β regulation of mTOR to induce autophagy, [48].

Our observed lack of LKB1 expression changes supported an alternate upstream mTOR inhibitor. To this effect, a third regulator of AMPK-mTOR signaling is CaMKK-β [31]. Research has shown more specifically that CaMKK-β is an upstream kinase involved in activating AMPK when a cell is under stress, exhibiting elevated levels of intracellular Ca. Others have shown a statistically significant decrease in phosphorylated AMPK was observed when STO-609, a known CaMKK-β inhibitor, was placed on cells undergoing stress [49]. Recent research has found conflicting data with respect to the effects CsA can have on CaMKK-β regulation of AMPK and autophagy. Research in prostate cancer cells has found that CsA activates CaMKK-β, thus resulting in the downstream effect of mTOR inhibition and induction of autophagy [48]. By comparison, in hippocampal cells, CsA treatment did not result in CaMKK-β expression changes [44]. Our data aligns with the latter study, in that CsA-treated LEC were still induced to undergo autophagy even when CaMKK-β was inhibited, as indicated by increased LC3-II expression (**Figure 6**).

In order to demonstrate that AMPK was not directly inhibiting mTOR following CsA treatment, Compound C was used in conjunction with CsA treatment. Compound C has been proven to be a potent reversible competitive inhibitor of AMPK and has been used in cancer treatment studies [50]. Use of Compound C did not prevent autophagy in CsA-treated LEC. This indicates that AMPK does not appear to be responsible for mTOR inhibition and induction of autophagy in CsA treated LEC.

Activated AKT can indirectly stimulate mTOR to negatively regulate autophagy via two mechanisms, involving either PRAS40 or TSC2 [28, 29]. Thus, a reduction in AKT expression is expected to result in autophagy. We have previously shown that AKT is increased in cataract and PCO samples indicating this protein plays an important role in LEC growth and survival [51]. Pharmacologic inhibition of AKT decreased LEC viability and decreased *ex vivo* PCO formation [51]. As demonstrated in **Figure 8**, a significant reduction in AKT is observed within 24 hours of CsA treatment. This, in conjunction with our previously published data, demonstrates that pAKT inhibition reduces cellular viability and supports a functional role for the AKT pathway in CsA-induced autophagy. Future experiments should focus on establishing the AKT substrate in order to target where AKT is specifically working. To confirm the importance of AKT as an effector protein involved in mTOR-regulated autophagy, the phosphorylation status of TSC2 and PRAS40 in CsA-treated LEC should be evaluated.

Endoplasmic reticulum stress has a proven role in autophagy, and has recently been shown to regulate, in part, AKT-mTOR signaling [34]. Following exposure to ER stress inducers, embryonic fibroblasts demonstrated decreased AKT-mTOR activity and

had increased expression of the autophagy marker LC3-II [34]. Similar to our study, AMPK did not appear to be involved in ER-stress induced autophagy [34]. Multiple chemical compounds, including rapamycin and tunicamycin, can induce ER stress by disturbing the ER milieu or hampering protein modification and transportation [36]. In support of the hypothesis that the ER is involved in CsA-induced autophagy, we have demonstrated that cultured LEC treated with CsA have accumulation of ubiquitinated proteins; co-treatment with CsA and the autophagy inhibitor 3-MA, resulted in a reduction in these ubiquitinated proteins (**Figure 9**). Treatment with CsA and salubrinal, a selective phosphatase inhibitor that protects cells from ER stress, also reduced ubiquitinated protein accumulation and maintained cell density (**Figures 9 and 10**). This data confirms that ER stress contributes to CsA-induced autophagy.

Collectively, these data show that CsA-induced autophagy is regulated through the mTOR pathway via ER stress and pAKT. These experiments demonstrate that AMPK likely plays little to no role in CsA-induced LEC autophagy. It is important to note, that based on our data, changes in the activation of AMPK following CsA treatment cannot be completely ruled out. Due to limitations in antibody cross-reactivity, we were unable to directly evaluate the role of AMPK; only indirect evaluation of AMPK was performed. It is possible that CsA alters AMPK expression and activity and as such, future experiments should confirm that AMPK expression does not change following CsA treatment in LEC. However, even if AMPK activity was altered by CsA, it is unlikely that AMPK would play a role in CsA-induced autophagy since inhibition of AMPK could not mitigate the effects of CsA in treated LEC. In contrast, pAKT expression was significantly reduced in

CsA-treated LEC suggesting a role for AKT in mTOR-regulated LEC autophagy. This research also demonstrated that misfolded proteins accumulate within CsA-treated LEC, as observed via increased ubiquitination; this effect was mitigated with salubrinal treatment. Further, use of 3-MA reduces CsA-induced ubiquitination indicating that ER-stress plays a role in CsA-induced autophagy in the lens.

Induction of autophagy in CsA-treated LEC resulted in increased cell death by a mechanism that has not been fully explained. Preliminary, unpublished data indicates that autophagy dysregulation results in activation of apoptotic signaling cascades. It is expected that this mechanism of cell death would be beneficial in the intraocular environment by having little to no effects on surrounding tissue. Other forms of cell death, like necrosis, cause an inflammatory reaction resulting in destruction of surrounding tissue [52]. Induction of intraocular inflammation can be detrimental to vision, thus controlled intraocular cell death is thought to be beneficial with respect to reducing PCO. The innocuity of CsA to the intraocular environment is supported by the literature [21, 22].

A recognized limitation of most *in vitro* studies is the absence of naturally occurring processes, such as the release of growth factors, which may influence cellular behavior. As previously mentioned, all outlined experiments were performed using canine LEC. This may be viewed as a limitation because primary human LEC were not feasible for experimentation. However, the use of canine LEC allows experiments to be performed in primary cell cultures which are more similar to native LEC than

immortalized LEC cultures. Available human LEC are typically immortalized and do not always fully recapitulate what is observed in primary cell lines.

Another limitation is that while we demonstrate a role for ER stress in autophagy through indirect changes observed with ubiquitin expression and treatment with salubrinal and 3-MA, specific ER stress markers were not evaluated. Thus, another area for future research is to confirm and further characterize the precise role ER stress plays in LEC autophagy. Markers of autophagy were not evaluated when performing the ER stress experiments. Future experiments should be performed treating LEC with salubrinal in the presence or absence of CsA with subsequent evaluation of LC3-II and p62 expression. It is expected that salubrinal treatment will reduce LC3-II expression and decrease in p62 expression, indicating normal autophagic flux even in the presence of CsA. Expression of LC3-II and p62 could also be evaluated for all experiments that were performed to determine the upstream regulation of mTOR. This would confirm if any alterations in autophagic flux were occurring, despite the use of inhibitors such as Compound C.

In conclusion, this data begins to characterize the mechanism by which CsA induces autophagy in LEC *in vitro*, specifically, via pAKT regulation of the mTOR pathway and induction of ER stress (**Figure 11**). Autophagy is a complex mechanism of maintaining cellular homeostasis. Use of drugs such as CsA hijacks the role autophagy plays in homeostasis, leading to altered autophagic flux, which may ultimately promote cell death. This work provides a major step forward in finding clinically useful therapies to prevent PCO. This data will influence the design of future *in vivo* treatment

methodologies for PCO prevention in post-surgical cataract patients worldwide. Future directions include additional research into appropriate vehicles in which to deliver the drug while preventing additional side effects in the surgical routine. This research holds to the promise that one day post cataract surgery blindness due to PCO will be a complication of the past, leading to more people of the world having long term benefits clear vision post cataract surgery.

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