Chapter 6

New Biomarkers in the Retina and RPE Under Oxidative Stress

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Additional information is available at the end of the chapter

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1. Introduction

Age-related macular degeneration (AMD) involves progressive cell death of post-mitotic retinal pigment epithelial (RPE) cells, which adversely affects rod and cone survival. RPE cells are exposed to chronic oxidative stress, including constant exposure to intense light and increased reactive oxygen species (ROS) from mitochondria due to high levels of oxygen consumption. However, understanding how oxidative stress causes phosphoproteomic alterations that initiate death signals in the RPE is elusive. Lack of such knowledge is an important problem, because, without it, acquiring the ability to modulate cellular protection is highly unlikely.

The RPE, located between photoreceptors and choroid, is in a unique position to mediate the transport of nutrients, oxygen, and retinoid from blood to photoreceptors. For continued vision, the RPE is required for retinoid recycling and phagocytosis, which initiates accumulation of retinoid byproducts that eventually leads to apoptosis. Continuous exposure to light causes the RPE to consume a large amount of oxygen in order to complete the complex processes of nutrient transport, phagocytosis, and the visual cycle. It is still not known why the initial retinal degeneration occurs and how the degenerative processes progress as a result of oxidative stress. Adaptation to changes in oxidative environment is critical for the survival of retina and RPE cells. Clinical trials demonstrated a significant reduction toward retinal degeneration upon intake of antioxidants that include lutein, zeaxanthin, zinc, vitamin C, and vitamin E. Oxidation of polyunsaturated fatty acids (PFA) and photosensitizers in the RPE induces reactive oxygen species (ROS) production upon exposure to visible light. Hydrogen peroxide (H_2O_2) is generated in the RPE during phagocytosis of the photoreceptor outer segment, and it has been used as a direct oxidative-inducing reagent to initiate cellular oxidative stress. Understanding of molecular mechanisms that mediate oxidative stress-induced proteome changes in the RPE may

provide insight into the pathogenesis of retinal degeneration. In our study, comparative and differential proteomics have been applied to investigate global changes in the RPE and retina proteome due to oxidative stress induced by light or H_2O_2.

2. Phosphorylation signaling in the RPE

We have focused on understanding the cell death mechanism of the retina and RPE under oxidative stress [Chung et al., 2009; Lee et al., 2010a; Lee et al., 2010b; Zhang et al., 2010; Lee et al., 2011; Arnouk et al., 2011; Sripathi et al., 2011]. Our studies demonstrated that oxidative stress may trigger induction of anti-apoptotic erythropoietin, JAK2, and BCL-xL, as well as pro-apoptotic caspases. Oxidative stress also influences mitochondrial-nuclear communication by shuttling mitochondrial prohibitin. We examined whether phosphorylations of cytoskeletal and anti-apoptotic proteins, including vimentin, PP2A, and crystalline, regulate the initial protecting mechanism. The cytoskeletal network formed by filamentous proteins determines how retinal and RPE cells respond to their extracellular environmental stimuli that include oxidative stress.

Site-specific phosphorylations of crystalline and vimentin regulate protein interactions through positive or negative feedback mechanisms. The significance of proteomic studies is that they are providing a detailed apoptotic mechanism and new potential AMD-related biomarkers. The global phosphoproteome changes in RPE cells under oxidative stress will help to identify key elements of phosphorylation signaling that serve as a framework of biochemical pathways of AMD progression.

It is a critical question to understand the maintenance mechanism of appropriate phosphorylation signaling under chronic stress conditions. Oxidative stress may influence the expression of genetic risk factors, including complement factor H (CFH). Although the potential importance of protein phosphorylation/dephosphorylation as a therapeutic target has been appreciated, no detailed approach to date has been made targeting biomarkers in phosphoproteome to treat ocular diseases. For example, abnormalities in vimentin phosphorylation have been linked to neurodegenerative diseases, including AMD and Alzheimer's disease [Madigan et al., 1994; Yen et al, 1983; Yu et al., 1994], but the phosphorylation network mechanism in RPE cells under oxidative stress remains largely elusive.

Regulation of the phosphate on/off switch is a central tool for RPE cells to survive under stress conditions. Unbiased approach to determine phosphorylation sites and kinetics will help to understand the complex apoptotic network and epigenetic controlling mechanism of RPE death. A general proteomic approach that includes protein identification by mass spectrometry has been used routinely for the last decade, however, RPE phosphoproteomics remains challenging due to the complexity, substoichiometry (less than 50% phosphorylated), and kinetics (short vs. long time frame). Understanding phosphorylation in AMD is not clear, because protein phosphorylation is a very dynamic process, while the aging process is slow. Recent animal models exhibiting some of the features of human AMD

are available, but most of these models do not represent the full spectrum of pathological changes observed in human AMD. Animal models that mimic the complex and progressive characteristics of AMD are extremely valuable for studying the pathogenesis of AMD and testing different treatment modalities.

Phosphoproteins have been studied using chemical and affinity-based methods [Zhou et al., 2008; Tao et al., 2005]. However, the rapid and dynamic nature of the underlying changes and the low abundance of phosphoproteins reflecting their substoichiometry present challenges to the quantitative study of the phosphoproteome. Thus, the isolation of the phosphoproteome or phosphopeptidome represents a potentially advanced step in this analysis. We introduced a system-wide, unbiased, and high-throughput approach to investigate global phosphoproteome of oxidative stress-induced or aged RPE and retinal cells using phosphoproteome enrichment and labeling method. Phosphoprotein-enriched extracts from human RPE cells under stress were separated by two-dimensional (2D) electrophoresis. Serine, threonine, and tyrosine phosphorylation were visualized by 2D phospho-Western blotting and specific phosphorylation sites were analyzed by tandem mass spectrometry. We examined phosphoproteome changes under oxidative stress *in vitro* and aging-induced phosphoproteome *in vivo*.

Our results suggest a positive correlation between early biomarkers of phosphoproteome under oxidative stress and RPE proteins from AMD patients. The outcome of the current work is the initial delineation of the underlying physiology of oxidative stress-mediated phosphorylation signaling in RPE apoptosis. In addition, our study suggests a stimulus for understanding oxidative stress-induced cytoskeletal changes and the aggregate formation mechanism by phosphorylations. As a consequence, an effective therapeutic approach and animal model based on the modulation of phosphorylations are expected to result.

Recently, pioneering proteomic profiling studies revealed protein expression changes in human RPE, drusen, and lipofuscin [West et al., 2001; Crabb et al., 2002; Schütt et al., 2007]. Other studies compared native differentiated to cultured dedifferentiated RPE cells [Alge et al., 2003; Alge et al., 2006]. Proteomics proved to be a useful tool in delineating changes in RPE with the progressive stages of AMD [Nordgaard et al., 2006; An et al., 2006; Norgaard et al., 2008], and diabetes [Decanini et al., 2007]. Also, proteomic tools were used to study changes in the vitreous humor associated with diabetic retinopathy [Kim et al., 2007] and retinal proteins in glaucoma model [Tezel et al., 2005]. Since oxidative stress is implicated in the etiology of several RPE diseases that include AMD, identification of molecular mediators and early signaling events under chronic stress is a crucial step for understanding cell death mechanism and retinal degeneration. However, limitations of proteomic approach, including minor proteins with low concentration, hydrophobic membrane proteins, reproducibility, and time and labor demanding processes, exist as huddles to get comprehensive details on the molecular mechanism. Moreover, there is no comprehensive database that could support the analysis of protein phosphorylation in human RPE from the standpoint of spatial and temporal changes.

Phosphorylation of specific amino acids, including serine, threonine, and tyrosine, is a significant modulator of protein function that regulates subcellular localization, protein–protein interaction, conformational change, and signal transduction. Charge changes by phosphorylation creates a protein switch mechanism, allowing reversible phosphorylation to modify intracellular signaling in response to specific microenvironmental and genetic conditions and thereby act as a basic survival tool. However, the rapid and dynamic nature of the underlying changes and the low abundance of phosphoproteins reflecting their substoichiometry present challenges to the quantitative study of the phosphoproteome. The isolation of phosphoproteome or phosphopeptidome represents a potential challenge. Our data and other evidence suggest that phosphorylations are involved in apoptotic or protecting signaling under oxidative stress [Chung et al., 2009; Zhang et al., 2010; Arnouk et al., 2011; Lee et al., 2011; Sripathi et al., 2011].

Proteomic study provides direct evidence and a molecular basis for phosphorylation signaling, cytoskeletal reorganization, and apoptotic mechanisms of AMD [Nordgaard et al., 2006; Crabb et al., 2002; Ethen et al., 2006; An et al., 2006; Warburton et al., 2007]. A detailed proteome analysis of AMD supports our hypothesis that there is a positive correlation between RPE biomarkers under stress and AMD as shown in **Table 1**.

To address the issue of phosphorylation in cytoskeletal reorganization, we examined morphologic and cytoskeletal changes of RPE cells under oxidative stress *in vitro*. The apoptotic cell surface changes initiate the protrusion, develop bubble-like blebs on their surface, and continue phagocytic alteration shown as the membrane disruption. In the middle stage, cells showed apical, basal and surface changes such as dense projection of microvilli. In later stage, cells broke into small, membrane-wrapped fragments.

To examine potential markers corresponding cytoskeletal reorganization under oxidative stress, we examined vimentin, an intermediate filament protein shown in both control and AMD drusen [Crabb et al., 2002]. ARPE-19 contains retinal G-protein receptor (RGR) and peropsin as light detecting chromophore. Previous observation of vimentin in RPE cells derived from human choroidal neovascular membranes in AMD, as well as in drusen and melanolipofuscin, supporting our choice of phosphoproteome biomarkers in RPE cells under stress [Schlunck et al., 2002; Crabb et al., 2002; Warburton et al., 2007]. Previously, proteomic analysis of the retina revealed that the expression levels of vimentin and PP2A are significantly increased when C3HeB/FeJ mice (rd1 allele, 12 weeks, photoreceptor degenerated) are exposed under continuous light for 7 days compared to a condition of 12h light/dark cycling exposure [Zhang et al., 2010]. When melatonin is administered to animals while they are exposed to continuous light, the increased levels of vimentin and PP2A return to a normal level. Further, vimentin has been shown to be a target of PP2A that directly binds vimentin and dephosphorylates it. Vimentin is present in all mesenchymal cells, and often used as a differentiation marker. Like other intermediate filaments, vimentin acts to maintain cellular integrity; however, vimentin may play a role in RPE survival by phosphorylation.

AMD RPE proteome [1]	**AMD drusen** [2]	**RPE blebs** [3]	***RPE proteome under OS in vitro*** *[4]*	***RPE proteome under light in vitro*** *[5]*
ATP synthase β CRABP1 CRALBP Crystallin αA Elf4H GST π HSC 70 HSP 60 HSP 70 Mt HSP 75 Pyruvate kinase VDAC 1	Annexin 5 Clusterin Complement component 9 Crystallin αB Crystallin βA3 Histone H2A2 Serum albumin TIMP3 Vimentin Vitronectin	Annexin 5 Cytoskeleton-associated protein 4 Desmin EF 1 GST GTPase Rab 14 HSP 70 9B Keratin 7, 18 Lamin A/C MMP-14 Peroxiredoxin 5 Thioredoxin reductase 1 Tubulin VDAC3	*Annexin 5* *BUB3* *EF2* *GST π* *Guanine binding protein* *HSP 90 α* *HSP β1* *Lamin A/C* *Peroxiredoxin 1, 2* *Phosphomevalonate kinase* *Plasminogen activator-inhibitor 1* *Prohibitin* *Pyruvate kinase* *Retinol binding protein* *RPE65* *Thioredoxin-dependent-peroxide reductase 3* *VDAC2*	*Actin* *ATP synthase* *CRALBP* *Creatine kinase* *Crystallin αA* *Crystallin αB* *Crystallin βB* *Crystallin γ* *G binding protein β1* *HSP 70* *HSP 90* *HSP β1* *IRBP* *Plasminogen* *Protein kinase 4* *Pyruvate kinase* *RPE65* *Tubulin α1B* *Tubulin β2*
AMD retina proteome [6]	**RPE secretome** [7]	**AMD RPE secretome** [7]	**Melanolipofuscin** [8]	***Retina proteome in vitro under OS*** *[9, 10]*
CRABP Crystallin αA Crystallin αB HSP 60 HSP70 Tubulin α VDAC1	Annexin 5 Caspase 5 CFB CFH Complement C HSP 47 Laminin MMP2 Prasminogen activator inhibitor 1	CFB CFH Collagen α Complement C Custerin Galectin 3 BP MMP2	Annexin 5 ATP synthase Crystallin αB G binding protein HSP 60 HSP 70 Prohibitin RBP 3 RDH 11 RDH 5 RGR RPE65 Tubulin α Tubulin β VDAC 1, 2, 3 Vimentin	*Bcl-xL* *Caspase-3* *c-FOS* *Crystallin αB* *Crstallin βB* *Crystallin βS* *Crystallin γB* *Crystallin γF* *EPO* *EPOR* *G binding protein* *Jak2* *Peroxiredoxin 2* *Peroxiredoxin 6* *PP2A* *Tubulin β* *Vimentin*

[1] Nordgaard et al., 2006 [2] Crabb et al., 2002 [3] Alcazar et al., 2009 [4] Arnouk et al., 2011, [5] Lee et al., 2011, [6] Ethen et al., 2006 [7] An et al., 2006 [8] Warburton et al., 2007 [9] Chung et al., 2009 [10] Zhang et al., 2010

Table 1. Comparison of AMD proteome vs. retina/RPE proteome changes under oxidative stress. Early biomarkers from our studies are in Italics.

Vimentin is a major component of intermediate filament that spread throughout the cell and serve as signal transducer conveying mechanical and molecular information from cell surface to nucleus and intracellular compartments. Modifications of vimentin are essential reactions of filament dynamics. Changes of vimentin phosphorylation are directed to reorganization of the intermediate filament network and altered function of RPE cells. Hyperphosphorylation of vimentin can disassemble intermediate filament into soluble monomeric form. Our results suggest that vimentin is critically involved in the process of light-induced damage in RPE cells. By reducing the light-induced post-translational modification of vimentin, PP2A may assist to maintain the proper filament network in RPE cells.

Next we tested the hypothesis that oxidative stress may induce protein phosphorylation, including vimentin. Vimentin phosphorylation at S38 is downregulated under stress and is a potential PP2A specific site for dephosphorylation. Phosphorylation at S38 suggests a potential antiapoptotic role when considering increased Bak under oxidative stress. Phosphorylation at S55 under stress condition is upregulated compared to β-actin control. We examined whether normal light- induced phosphoproteome was altered in RPE cells. To investigate RPE phosphoproteome, we used 2D electrophoresis to separate proteins from cells incubated under light (7000 lux) or dark conditions for 1 hour. RPE proteins induced by light were visualized using mass spectrometry-compatible silver staining. MALDI-TOF-TOF mass spectrometry analysis revealed that a large proportion of the proteins up-regulated first and foremost by light in the RPE belong to the crystallin family. Our data demonstrated that crystallins were upregulated under oxidative stress in bovine primary RPE cells. Serine phosphorylation of crystallin was markedly increased in light exposure, as shown by pSer-specific Western blotting. Notably, opposite effects on serine and tyrosine phosphorylation may yield positive or negative regulation with the same stimulus [Taniguchi et al., 2006]. Enzymes associated with phosphorylation or energy metabolism, including Ser/Thr protein kinase 4 (STE20) and ATP synthase, were downregulated under intense light. The most abundant light-induced upregulated phosphoproteins were crystallins, including αA and αB crystallins. Our results may suggest that phosphorylation of crystallin is critical to maintain a chaperone function in RPE cells. Phosphorylations at serine residues were next to proline or near proline. The results of phosphoproteome enrichment experiment were confirmed using an affinity-based phosphopeptide-enrichment strategy. In this experiment, in-solution trypsin digested peptides were concentrated using a metal (Ga^{+3}) based column and analyzed by tandem mass analysis.

Phosphorylation-dependent signaling might differ between tissues, and might reflect the microenvironmental diversity among different tissues, including differences in stress stimuli. Phosphoproteome change is an indication of an early signaling event under oxidative stress conditions in RPE. Increased phosphorylation of crystallins, in particular, might suggest a stress-response role for these proteins in the RPE. Accordingly, crystallin phosphorylation may provide an anti-apoptotic signal to attenuate oxidative stress-induced degenerative pathway as early signaling event. However, accumulated phoshprylated crystallins may lead to pathological mechanism [den Engelsman et al., 2005]. In this context,

stress-induced up- or down-regulation of crystallins is known to occur in various diseases or age-related conditions [Nordgaard et al., 2006; Ethen et al., 2006]. The functional relationship between heat-shock proteins and cytoskeletal proteins, such as intermediate filaments, vimentin and actin, is well documented [Clark et al., 2000; Xi et al., 2003]. N-terminal phosphorylation of heat-shock proteins is correlated with the stabilization of cytoskeletal elements.

Our studies support the hypothesis that a progressive accumulation of oxidative damage is a fundamental mechanism involved in RPE cell death. Oxidative stress occurs when free radicals produced in RPE cells are not completely modulated by the appropriate endogenous defense systems. Phospholipids are a major component that can be targeted by excess oxidative stress. Lipid peroxidation plays an important role in initiating and mediating phosphorylation and cell death signaling. Establishing the involvement of lipid changes in phosphorylation signaling has not been an easy task. The lipids of cellular membranes serve roles in controlling the structure and fluidity of the membrane, and as signaling molecules that modifies protein functions. The lipid binding assay demonstrated subcellular communication between mitochondria and the nucleus under oxidative stress. The changes in the expression and localization of p-crystallin and p-vimentin triggered by reactive oxygen species are crucial for RPE integrity.

Recently, crystallin location and function in the retina and RPE have been described [Sakaguchi et al., 2003; Organisciak et al., 2011; Zigler et al., 2011; Sreekumar et al., 2010; Gangalum et al., 2011]. αA- and αB-crystallin are located in the ganglion cell layer and photoreceptor layer, whereas β-crystallin is detected in all nuclear layers of the retina [Xi et al., 2003]. The functional roles of crystallins as chaperones, anti-apoptotic proteins, or signal transducers in the retina have also been described [Xi et al., 2003; Sakaguchi et al., 2003; Kapphahn et al., 2003; Kim et al., 2007]. Phosphorylated αB-crystallin directly interacts with Bax and caspase-3 to suppress their pro-apoptotic action, and thus exerts a cytoprotective effect in the retina [Kim YH et al., 2007]. Light-induced up-regulation of αA-crystallin and increased phosphorylation of αA-crystallin in the aged retina have been reported [Kapphahn et al., 2003]. The specific function of each crystallin, particularly α/β-crystallin in the RPE, is still largely unknown, although levels of αB-crystallin are increased after heat shock and oxidative stress, and αB-crystallin immunoreactivity has been found in both rod outer segments and the RPE after light exposure [Sakaguchi et al., 2003].

3. Early proteome changes under oxidative stress

Two-dimensional differential gel electrophoresis (2-D DIGE) is an advanced proteomic technique that labels minor protein samples with fluorescent dyes before 2-D electrophoresis. It enables accurate analysis of differences in protein concentrations between samples. DIGE method reduces experimental variations and technical errors. It is possible to separate up to three different samples within the same 2-D gel using three different fluorescent molecules. However, there are limitations of DIGE methods also, including covalent modifications on cysteine or lysine.

We used two different biological models that include primary bovine RPE cells and human RPE cell line D407, to uncover differential biosignatures under oxidative stress [Davis et al., 1995]. We employed a combination of proteomic technologies, including sensitive fluorescent labeling and TOF-TOF mass spectrometry analysis with high mass accuracy and low tolerance in the range of 50 ppm (0.05 Dalton). Identified proteins were confirmed by quantitative Western blotting. We observed expression changes of cellular signaling related molecules, including intermediate filament, retinoid metabolism, energy metabolism, and antioxidant proteins in bovine RPE cells. Several intermediate filament components, including neurofilament H, M, L proteins and glial fibrillary acidic protein, were up-regulated in bovine RPE cells. Intermediate filament proteins are cytoskeletal components that form fibrils with an average diameter of 10 nm. Neurofilament H, M, and L proteins are specifically expressed in neurons. Glial fibrillary acidic protein is a biomarker in glial cells. Previous studies showed that environmental changes, such as culture conditions, could induce dedifferentiation of RPE cells and give rise to mesenchymal-like cells.

Proteome changes related to energy metabolism were also observed under oxidative stress in bovine RPE cells. Pyruvate dehydrogenase E1 transforms pyruvate into acetyl-CoA that is used in the citric acid cycle to generate ATP. Pyruvate kinase M1 transfers a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), to synthesize ATP and pyruvate. Up-regulation of both enzymes may indicate higher energy consumption to compensate oxidative stress-induced faster turnover of proteins in the RPE.

In RPE D407 cells, several molecular chaperones were altered as a result of H_2O_2 treatment. Hsp 90α and Hsp β1 exhibit general protective chaperone properties such as preventing unspecific aggregation of non-native proteins. Calreticulin, up-regulated in bovine RPE cells, is a multifunctional protein that binds to Ca^{2+} and misfolded proteins to export them from the endoplasmic reticulum (ER) to the Golgi apparatus. Elimination of misfolded proteins in the ER affects cellular homeostasis and survival, so chaperone function of heat shock proteins is indispensible under stress conditions.

Retinoid metabolism in primary bovine RPE cells is altered under oxidative stress conditions. Photosensitivity and the steady state of retinoid concentrations are controlled by regeneration of 11-*cis*-retinoid, which is called the visual cycle. RPE65, a peripheral membrane protein of RPE cells, is an all-*trans*-retinyl ester isomerohydrolase. Indeed, we showed that RPE65 in bovine RPE is up-regulated under oxidative stress. Our data demonstrated that RPE65 is cleaved into a 45 kDa and 20 kDa truncation forms under oxidative stress. This data indicates that oxidative stress can influence the visual cycle by up-regulation of RPE65 and cleavage of this protein. Further studies of whether this truncated form might be a biomarker of oxidative stress are currently under investigation. It seems that immortalized human RPE cells suppress RPE65 expressions as a basic regulatory mechanism for the visual cycle that is not essential for survival. Prohibitin, a mitochondrial chaperone involved in oxidative stress and aging, was found to be downregulated under oxidative stress in primary bovine RPE cells.

Antioxidant proteins were upregulated under oxidative stress. Peroxiredoxin is a ubiquitous family of antioxidant enzymes that can be regulated by changes of redox potential in the cell. Thioredoxin-dependent peroxide reductase is an antioxidant enzyme that provides protection mechanism against oxidative stress. It reduces H_2O_2 by hydride provided by thioredoxin, thioredoxin reductase, and NADPH. Upregulation of antioxidants can regulate redox homeostasis in RPE cells and thus protect cells from oxidative stress-induced damage.

Both biological models using primary bovine RPE cells and immortalized D407 cells have advantages and limitations. We observed differences in protein expressions correlated with oxidative stress in two biological models used in our study, which can be attributed, at least partially, to the inherent differences in two different RPE cell types. It has been reported that cytoskeletal remodeling and cell survival factors are differentially expressed in primary culture and immortalized human RPE cells. Dedifferentiated, immortalized human RPE cells results in down-regulation of specific proteins associated with retinoid metabolism. At the same time, they induce the differential expressions related with cytoskeletal organization, cell shape, migration, and proliferation. Thus, we speculate that two different proteome list in two different systems such as primary bovine vs. immortalized human RPE cells is due to different cell type and not due to species difference or detection system.

Oxidative stress alone is the major risk factor of retinal degeneration compared to genetic risk factors. Furthermore, oxidative stress may influence the expression of genetic risk factor, such as complement factor H (CFH). Cellular processes induced by oxidative stress in RPE cells and the molecular mechanisms under the oxidative stress that contribute to retinal degeneration are not clear at this point. We revealed several expression changes in the RPE proteome that is induced by H_2O_2 treatment. Targeting early signaling proteins is a useful therapeutic strategy for the treatment of degenerative diseases of the retina and the RPE.

4. Prohibitin as a new oxidative stress biomarker

It is estimated that every ten minutes in the USA someone becomes blind. More than 4 million Americans have age-related macular degeneration (AMD), the most common cause of legal blindness in those older than fifty five. Even though the role of abnormal angiogenesis in the pathogenesis of AMD has been recently established, we still do not know why the macular deteriorates and why AMD progresses. Our proteomic experiments demonstrate that prohibitin is a shuttling protein primarily expressed in mitochondria, however, it translocalizes to the nucleus under oxidative stress. The extent of prohibitin localization in mitochondria inversely correlates with levels of oxidative stress. Our knockdown approach suggests that prohibitin may have the anti-apoptotic function through Bcl-xL and mitochondrial DNA binding mechanisms. Our data further suggests that specific lipid-binding mechanisms involving altered cardiolipin interactions might be the key element to determine prohibitin shuttling between mitochondria and the nucleus. Mitochondrial dysfunction is associated with AMD and our preliminary studies also suggest specific pathophysiological mechanisms involving altered mitochondrial disruption.

Prohibitin is involved in the alternative pathway of complement C activation. However, the regulatory mechanisms are elusive. Prohibitin is subjected to various post-translational modifications under growth factor stimulation and oxidative stress. Prohibitin induces a conformational change that enhances activation of C3.

Prohibitin is originally known as an anti-proliferative protein [McClung et al., 1989]. It is highly conserved from bacteria to human [Asamoto and Cohen SM, 1994], but its function is not clearly understood. Prohibitin was proposed to have a role as a cell cycle inhibitor [Nuell et al, 1991; McClung et al, 1995], a transcriptional regulator [Wang et al, 1999a; Wang et al, 1999b], an inflammatory modulator [Sharma and Qadri, 2004], a plasma membrane receptor [Kolonin et al, 2004], and a mitochondrial chaperone [Nijtmans et al, 2000; Artal-Sanz et al, 2003; Steglich et al, 1999]. Its chaperone function in mitochondria has recently received more attention as a modulator that responds to oxidative stress [Coates et al., 2001]. Prohibitin is involved in apoptosis and aging by stabilizing newly synthesized respiratory enzymes [Bourges et al, 2004; Nijtmans et al, 2002], however, its presence and function in the eye has never been studied. Our studies demonstrate that prohibitin is a novel protein that shuttles between mitochondria and the nucleus as an anti-apoptotic chaperone as well as a transcriptional regulator under oxidative stress [Arnouk, et al., 2011; Lee et al., 2010; Srinivas et al., 2011].

Prohibitin undergoes phosphorylation at multiple residues under various cellular condition or in response to various stimuli. Phosphorylation of prohibitin facilitate its interaction with SH2 domain containing phospho tyrosine phosphatases Shp1 and Shp2. In a separate study, prohibitin was identified as a substrate for Akt and it has been shown that Akt induced prohibitin phosphorylation at threonine258. While these studies have clearly shown that prohibitin is phosphorylated at multiple residues, however, conditions under which these residues are phosphorylated and functional consequences are not clear. Prohibitin binding proteins were apoptosis related (Bcl-2 like protein), cholesterol homeostasis (PI4K5, diacylglycerol kinase, apolipoprotein B48 receptor, VLDLR), nucleotide binding proteins (guanine exchange factor, endonuclease, cell cycle regulator) and a complement component (Table 2).

5. Anti-apoptotic prohibitin function in mitochondria

We performed loss of function studies using prohibitin-specific small interfering RNA (siRNA) to demonstrate an increase of apoptotic signaling when prohibitin was knockdown. We tested two siRNA constructs that inhibit prohibitin expression compared to a control siRNA that has a random sequence. A solvent vehicle was used as the second negative control also. We chose conditions when prohibitin expressions were reduced by 80-90% compared to random sequence control. We examined whether prohibitin knockdown activates pro-apoptotic AIF, BAK, and caspases. Prohibitin knockdown may control other anti-apoptotic molecules also. Indeed, pro-apoptotic AIF, caspase-9, and BAK increased under prohibitin knockdown condition whereas anti-apoptotic Bcl-xL decreased. Also we examined decreased prohibitin condition cleaves poly (ADP ribose) polymerase (PARP) as a

Prohibitin binding proteins	MW/pI	Function
Vav-1 guanine exchange factor	98 kDa/6.2	proto-oncogene
ATP binding cassette 4	66 kDa/6.9	regulator of cAMP level
DEAD box 32	84 kDa/5.0	RNA helicase
epithelial cell transforming 2	104 kDa/7.5	oncogene
guanine exchange factor	119 kDa/5.9	activation of GTPases
Transglutaminase	93 kDa/6.5	protein aggregation
phosphoinositol-4-phosphate 5 kinase	45 kDa/9.6	epithelial cell morphology actin polymerization
Nei Endonuclease VIII like protein 1	37 kDa/9.2	DNA excision repair
Bcl-2 like protein 14	37 kDa/7.7	apoptosis facilitator
calpastatin	76 kDa/4.9	calpain inhibitor
complement component C6	105 kDa/6.7	membrane complex
cell cycle regulator Mat89Bb homolog	80 kDa/6.2	nucleus-centrosome coupling
diacylglycerol kinase zeta	103 kDa/9.8	phosphatidic acid synthesis
Apolipoportein B48 receptor-like	109 kDa/4.4	carrying cholesterol to tissues
very low density lipoprotein receptor	93 kDa/4.7	regulator of cholesterol levels

Table 2. Prohibitin binding proteins determined by immunoprecipitation.

pro-apoptotic marker because PARP cleavage is an early marker of caspase activation. Transfection of siRNA of prohibitin into ARPE-19 cells resulted in increased cleavage of PARP which represents that an apoptotic signal is induced when prohibitin is down-regulated.

Next we examined whether caspases are also activated as essential markers in apoptosis. The activation of caspase-9, one of the upstream regulators in apoptosis, showed early apoptotic signaling under prohibitin knockdown condition. Our results suggest that knockdown of prohibitin increases caspase-dependent apoptotic signaling. This data implies that prohibitin may act as an anti-apoptotic protein in mitochondria by forming a functional complex with other apoptotic proteins. Prohibitin concentration is inversely correlated with the initiation of apoptosis. Then we asked whether decreased prohibitin disrupts the filamentous reticular network of mitochondria resulting in fragmented mitochondria. Immunocytochemical analysis showed that prohibitin knockdown led to accumulation of fragmented mitochondria. Then we asked whether expression and localization of prohibitin is altered under stress condition.

Emerging evidence suggest that oxidative stress and associated local upregulation of alternative pathway of complement may play a critical role in the progression of AMD. However, the link between oxidative stress. Altered prohibitin function may be a connecting link between oxidative stress and complement activation in AMD. Our studies demonstrate that prohibitin expression in the RPE is altered under oxidative stress *in vitro* and *in vivo*. We tested an advanced proteomic approach using a 2D differential gel electrophoresis (DIGE) technique to label RPE proteome by fluorescent molecules to uncover new biomarkers

under stress conditions. We found that prohibitin is a chaperone molecule maintaining mitochondrial structure and function shown by immunoprecipitation and knockdown method.

We have been actively seeking early molecular signaling events under oxidative stress in the retina and RPE using a proteomics and metabolomics approach to identify new biomarkers. This approach demonstrated that prohibitin is involved in oxidative stress signaling *in vitro* and *in vivo* [Lee et al., 2010b; Arnouk et al., 2011; Srinivas et al., 2011]. Prohibitin was proposed as an anti-proliferative protein or a tumor suppressor forming a high molecular complex with prohibitin2 in mitochondria [Steglich et al., 1999]. Localization of prohibitin has been controversial, possibly being mitochondrial, nuclear, or cell surface [Wang et al., 2002; Rivera-Milla et al., 2006].

We examined the cellular localization of prohibitin which is a controversial question. Confluent ARPE19 cells were treated with 200 μM H_2O_2 for 24 hrs and prohibitin localization in response to oxidative stress was investigated using immunocytochemistry. Subcellular organelles and prohibitin were visualized by DAPI (nuclear DNA, blue, 369/460 nm absorbance/emission), MitoTracker (mitochondria, red, 578/599 nm absorbance/emission), and Alexa-488 (prohibitin, green, 495/519 nm absorbance/emission), by taking three color fluorescent images every hour. This kinetic assay confirmed that the signaling pathway was involved in prohibitin transit to the nucleus from mitochondria. H_2O_2 treated cells showed nuclear translocalization of prohibitin, whereas untreated cells showed localization in mitochondria. After 8 hrs, prohibitin showed an accumulation in the nucleus as compared to the control group. After 24 hrs, more nuclear prohibitin was observed and prohibitin didn't show as much colocalization with mitochondria.

To answer whether prohibitin is modified under stress condition, we tested the correlation between oxidative stress and the ratio of soluble vs. membrane-binding prohibitin. Because prohibitin may exist in both detergent-resistant and soluble fractions, we tried to separate prohibitins by longer running time SDS-PAGE. Soluble prohibitin in the cytosolic fraction moved faster when compared to membrane-bound mitochondrial and microsomal prohibitin. Detergent-resistant, membrane-binding prohibitin showed slightly higher molecular weight when compared to soluble prohibitin. We found that the ratio of soluble prohibitin to membrane binding prohibitin decreased under oxidative stress. Under stress condition, soluble prohibitin decreased and membrane-binding prohibitin increased. To answer how long this trend would last under oxidative stress, we performed a kinetic assay under oxidative stress. After 6 hrs, the soluble prohibitin level returned to its control level. It is possible that the membrane-binding and soluble form exists in different subcellular organelles, so we next examined the subcellular localization of prohibitin by fractionation.

Membrane-binding prohibitin showed slightly higher molecular weight when compared to soluble prohibitin. We examined the correlation between oxidative stress and soluble vs. membrane-binding prohibitin and found that the ratio of soluble prohibitin to membrane binding prohibitin decreased under oxidative stress. Soluble prohibitin decreased and membrane-binding prohibitin increased under stress condition. Then we tested how long

this trend would last under oxidative stress. After 6 hrs, the soluble prohibitin level returned to its control level. It is possible that the membrane-binding and soluble form exists in different subcellular organelles, so we examined whether prohibitin binds a mitochondrial specific phospholipid, cardiolipin.

6. Prohibitin binds cardiolipin under oxidative stress

We asked whether prohibitin localization could be controlled by membrane lipid binding. We speculated that the localization and trafficking of prohibitin might be determined by mitochondrial-specific lipid such as cardiolipin. Lipid interaction assay demonstrated that mitochondrial prohibitin has a strong affinity at low cardiolipin concentration whereas nuclear prohibitin showed a much weaker affinity to cardiolipin, interacting at higher concentrations. Lipid analysis in ARPE-19 cells by mass spectrometry demonstrated that cardiolipin concentration decreased 20-70% under 200 μM H_2O_2.

This result suggests that membrane lipid and modification may determine prohibitin localization under oxidative stress. When cardiolipin concentration is low under oxidative stress, prohibitin moves to the nucleus. Then we tested whether prohibitin may have preference toward specific lipids when prohibitin is a limiting factor. Under normal condition, prohibitin from ARPE-19 cells demonstrated a strong interaction with phosphatidylinositol 3,4,5 triphosphate (PIP3), PIP2, PIP, and 3-sulfogalactosylceramide, but not with cardiolipin. Lipid binding affinity changed dramatically under oxidative stress. Prohibitin under stress showed a strong affinity toward cardiolipin, phosphatidylserine, and phosphatidic acid. This observation led us to conclude that prohibitin has an affinity towards negatively charged phospholipids, especially cardiolipin under oxidative stress.

To examine prohibitin response under oxidative stress in detail, we then examined protein expression level changes *in vitro* using ARPE-19 cells. We examined whether prohibitin level changes under H_2O_2 in time and dose dependent manner. Protein analysis by SDS-PAGE and Western blot demonstrated that prohibitin is down-regulated under oxidative stress (50-200 μM H_2O_2, 1-24 hrs). Cells treated with 200 μM H_2O_2 showed a 40% decrease of prohibitin, but the levels between treated groups in 24 hrs revealed no significant difference by t-test, indicating that decreased levels of prohibitin under oxidative stress is an acute process that occurs within 2 hrs. This result suggests that the nuclear prohibitin may not be a newly synthesized protein as there was no prohibitin increase for 24 hrs; instead the nuclear prohibitin might be derived from mitochondria since prohibitin was not colocalized with mitochondria after extended H_2O_2 treatment.

Our previous *in vivo* experiments suggest that prohibitin may respond differently in the retina and RPE under oxidative stress conditions, including diabetes and aging. To specify the location of prohibitin in cells, we separated subcellular fractions using serial centrifugations, specific detergents, and polyamines. Subcellular proteins were separated into soluble and insoluble nuclear and mitochondrial proteins as well as cytosolic and microsomal fractions. Denatured and native gel electrophoresis, followed by Western blot of subcellular organelle showed that prohibitin is widely distributed in cells but is centralized in mitochondria.

In mitochondrial fraction, prohibitin showed 32 kDa molecular weight and pI=5.6 as expected. Prohibitin was more acidic in the nucleus showing pI=5.3, possibly due to post-translational modifications such as phosphorylation. Mass spectrometry analysis revealed that S101 and S151 sites are phosphorylated. Cytosolic prohibitin showed very basic spots of pI=6-7 and higher molecular weight as shown in the box.

As each subcellular prohibitin showed different pI values in 2D electrophoresis, we examined potential post-translational modifications, including phosphorylation. Each fraction was separated by electrophoresis and prohibitin was visualized by Western blot using prohibitin and phospho-serine (pSer) antibodies. Only the nuclear fraction showed phosphorylated prohibitin shown by pSer Western blot. Mitochondrial, cytosolic, and microsomal prohibitin did not show phosphorylation. To confirm this data, we performed native gel electrophoresis and received the same result.

7. Neuroprotective erythropoietin (EPO)

Apoptosis is the primary mechanism that results in the abnormal death of photoreceptors, retinal ganglion cells (RGC), or retinal pigment epithelial cells (RPE) in degenerative retinal diseases, including age-related macular degeneration (AMD), retinitis pigmentosa (RP), and glaucoma. Light insults result in increased production of reactive oxygen and nitrogen species that include hydrogen peroxide and nitric oxide, which are involved in an early event of retinal degeneration. In response to intense or constant light triggers, neuroprotective proteins provide an antiapoptotic effect to protect retinal and retinal pigment epithelial (RPE) cells, however, the mechanisms remain elusive. Thus there is a critical need for understanding the intrinsic neuroprotective mechanisms that prevents apoptotic cell death. Our studies suggest that potential degeneration signaling may result from expression changes and phosphorylations of key proteins of EPO downstream. EPO down-regulates caspases and intense light up-regulates caspases, which imply that depleting EPO is one of the major light effects that can cause changes in protein expression through anti-apoptotic mechanisms.

We demonstrated that the retinal expression of EPO and subsequent phosphorylation of Jak2 and Stat3 are tightly linked to the circadian clock after oxidative stress and in anticipation of daily light onset. Our data suggest that the neuroprotective effects of EPO might be involved the regulation of apoptotic signaling molecules, including Bcl-xL, and caspase-3. In the RPE, NO was proposed as a secondary messenger in phagocytosis. Interdependent regulation of NO and EPO has not been examined, even though recent studies imply potential interactions of NO-EPO through the Hif1a pathway.

Retinal injury due to light occurs through oxidative mechanisms. Recently we demonstrated that the recycling reactions of 11-*cis*-retinal, called the visual cycle, is circadian coordinated to effectively protect the retina from the detrimental effects of light-induced and oxygen-dependent damage [Chung et al., 2009, Lee et al., 2010a]. Our data suggested that the retinal expression of EPO and its receptor (EPOR), as well as subsequent Janus kinase 2 (Jak2) phosphorylations, are tightly linked to a time window after oxidative stress and in anticipation of daily light onset. This is consistent with physiological protection against

daily light-induced, oxidative mediated apoptosis [Hardeland et al., 2003; Hrushesky 1985; Scheving et al., 1988; Smaaland et al., 1991; Smaaland et al., 1992].

We found that nitric oxide (NO) was generated in RPE cells under light exposure. NO is related to the inhibition of ischemic injury and plays a key role in the delayed cell death following transient retinal ischemia. Thus, NO is proposed as a neuroprotective molecule or neurotoxic reagent based on its local concentration. In the RPE, NO was proposed as a second messenger in phagocytosis. Interdependent regulation of nitric oxide and EPO has not been examined, even though recent studies imply potential interactions of NO-EPO through either HIF1a or arginine metabolism. Appropriate levels of nitric oxide (NO) may cause up-regulation of EPO/EPOR in RPE and thereby assist in limiting retinal degeneration.

EPO has been extensively studied for its neuroprotective role for the past 10 years, but the regulation mechanisms have not been understood at the molecular level [Brines and Cerami, 2005; Calapai et al., 2000; Jelkmann 2007; Kanaan et al., 2006; Kawakami et al., 2001; Li et al., 2007; Mauer 1965; Miyake et al., 1977; Yamaji et al., 1996; Zhong et al., 2007]. Previous approaches using the *in vivo* mice model was not satisfactory, because the EPO knockout mice model is lethal. No approach to date has been effective in moving beyond the addition of EPO *in vitro* and *in vivo*.

The *in vivo* erythropoietic effects of administered EPO are known to depend upon the time of administration [Wood et al, 1998; Bellamy et al., 1988]. Moreover, elevated EPO levels during the proliferative stage may contribute to neovascularization and accelerate pathological angiogenesis, in which EPO showed little therapeutic effect at later stages of retinopathy [Chen et al., 2008]. Light kills retinal cells. In response to light, neuroprotective proteins trigger an anti-apoptotic pathway in the retina to protect cells from light-induced oxidative stress. However, the mechanism that regulates expression of these retinal proteins remains elusive.

Erythropoietin (EPO) is an oxygen-dependent hematopoietic cytokine that stimulates the proliferation, differentiation, and survival of erythroid progenitor cells. EPO protects cells from neuronal damage, including experimental central nervous system models of hypoxic and ischemic insults [Jelkmann and Metzen, 1996; Jelkmann, 2005]. Oxygen dependent mechanisms are derived from models such as traumatic brain injury [Brines et al., 2000], spinal cord injury [Celik et al., 2002], Parkinson's disease [Kanaan et al., 2006], excitotoxicity [Kawakami et al., 2001; Morishita et al., 1997], oxidative stress [Calapai et al., 2000] and chemical neurotoxicity [Genc et al., 2001]. EPO can protect retinal ganglion cells (RGCs) from degeneration induced by acute ischemia reperfusion injury [Liu XT et al., 2006] and axotomy injury [Weishaupt et al., 2004].

EPO promotes survival of RGCs in a glaucoma mouse model [Zhong et al., 2007], and stimulates neurogenesis and post-stroke recovery [Tsai et al., 2006]. *In vitro* models reveal that EPO stimulates neuritic outgrowth by postnatal [Böcker-Meffert et al., 2002] and adult RGCs [Kretz et al., 2005]. EPO is produced in the retina in response to acute hypoxia via Hif-1α stabilization, which confers protection from light-induced retinal degeneration [Grimm et al., 2002]. The specific role of EPO was emphasized in that only EPO gene expression was

significantly affected among various angiogenic factors in Hif-1a-Like Factor (HLF) knockdown model [Morita et al., 2003].

However, controversial discussions of EPO in retinal neuroprotection exist. Understanding EPO function in pathological angiogenesis is critical to timing for intervention [Chen et al., 2008]. Our studies demonstrated that EPO and EPOR interactions represent an important retinal shield from physiologic and pathologic light-induced oxidative injury.

To test the hypothesis that EPO is regulated by light *in vivo*, retinas from mice subjected to normal 12 h light/dark cycle were accessed for EPO and EPOR protein content. Retinal EPO increased for 2 h after the light turned on and gradually declined in the late light phase. However, 2 h before the light returned, EPO increased. The 24 h pattern of EPOR followed a similar pattern to EPO. This result suggests that the endogenous circadian clock may regulate EPO and EPOR levels such that they increase just before light onset in anticipation of the daily light period (L/D 22 h). However, under constant dark condition (D/D 22 h), EPO/EPOR were not upregulated.

Our data demonstrated that the circadian organization of retinal EPO and EPOR elaboration is a potentially effective endogenous physiologic strategy for retinal protection from light-induced, oxygen-mediated damage. These findings are consistent with documented circadian regulation of the full range of oxidative damage protecting enzymes or antioxidants, including glutathione (GSH/GSSG).

Then, we tested the hypothesis that oxidative stress, including intense light, hypoxia, and hyperoxia, may induce EPO response in the retina *in vitro*. As early as 15 min after light exposure in retinal cells, EPO expression increased. Immunoblots showed that expressions of EPO increased 1 h after exposure to hypoxia and hyperoxia. In retinal and RPE cells, cells appeared morphologically viable, and no cell death was noticed for 6 hrs under hypoxic and hyperoxic conditions. Under oxidative stress, upregulation of EPO confers a neuroprotective function against retinal degeneration. Our aim was to explicate the role of the light and oxidative stress in the control of neuroprotective EPO expression in the retina and RPE.

We treated EPO (50-200 U) on rat retinal cells and human RPE cells *in vitro* under oxidative stress to examine its protective effect. EPO inhibited caspase-3 activation in retinal cells under stress, indicating that EPO is anti-apoptotic.

As an endpoint analysis, we performed a cell viability assay using a rat retina and human primary RPE cell culture treated with H_2O_2 as an oxidative stress model for a control experiment. At a concentration of 20 μM or higher for 3 hrs, H_2O_2 exerted significant toxicity to retinal cell cultures. H_2O_2-induced retinal cell death was reduced to 40, 46, or 43 %, respectively, by 30 min pretreatment of 50 units of EPO with 40 μM H_2O_2 for 6, 12, and 24 hrs. In contrast, only a mild protective effect (15%) was observed in the RPE exposed to H_2O_2 by EPO treatment in 24 hrs.

To see the protective effect in the RPE, a high dose of EPO (up to 200 U) was required, and was found mildly toxic to the RPE cells. Phase-contrast photomicrographs showed that no significant cell death occurred in sham-washed control or treated human RPE cells with 400

μM H_2O_2 for 12 h. Cell viability was assessed by the amount of lactate dehydrogenase (LDH) release measured from the medium of RPE cultures exposed to H_2O_2 for 3, 6, 12, and 24 hrs.

EPO is a powerful cytoprotective protein against apoptosis. However, elevated levels of EPO are found in the vitreous due to proliferative diabetic retinopathy [Katsura et al., 2005; Watanabe et al., 2005]. In addition, high dose of EPO increased the risk of cardiovascular disease and tumor growth [Singh et al., 2006; Bohlius et al., 2006].

These studies suggest a role of EPO in pathological retinal angiogenesis. The effects of EPO on angiogenesis are not well understood and the role of EPO in vascular stability is not clear. Understanding the functional role of EPO on angiogenesis is beneficial to patients with diabetic retinopathy (DR) and retinopathy of prematurity (ROP). EPO has been known to promote endothelial cell proliferation and vessel growth, however, the influence of EPO on apoptotic signaling and retinopathy is beginning to be understood. To determine the effects of EPO on vessel growth, we explored downstream regulators of EPO under normal physiological condition. VEGFR1 and angiotensin I/II as two major angiogenic factors were examined. VEGFR1 in the retina and angiotensin I/II in RPE increased after EPO treatment (100 U) in 6-12 h, respectively.

Our study suggests that the role of EPO in the development of initial vessel loss via VEGF/VEGFR/angiotensin signaling. This signaling is important in understanding retinal vessel loss that is initiated prior to neovascularization. The initial vessel loss determines the severity of neovascularization. VEGF was downregulated upon EPO knockdown in RPE cells, which may suggest that EPO and VEGF expression is regulated in parallel, and they may interact directly or indirectly.

EPO induces the proangiogenic phenotype in rat retina and human RPE cells. Addition of EPO correlates with the progression of VEGFR and AngiotensinI/II, which suggests that EPO might be an endogenous stimulant of vessel growth during retinal angiogenesis and in the development of neovascularization. Possibly, EPO mediates the renin-angiotensin system (RAS) which may promote neovascularization through local changes in blood flow and production of VEGF. EPO is considered as a proinflammatory agent since it triggers the expression of angiotensin I/II, which may act as an inflammatory agent by enhancing vascular permeability through VEGF. We examined whether the EPO-dependent pathway is up-regulated or down-regulated in various oxygen-imbalance conditions. Along with increased levels of EPO after light exposure, levels of rhodopsin in retinal cells increased as early as 15 min after 5000 lux light exposure in a time-dependent manner. RPE65 cleavage increased after 15 min exposure to 5000 lux light in the RPE. Immunoblots for Bcl-xL and c-Fos showed that the neuroprotective effects of EPO may involve upregulation of these early markers in stress-induced condition. Anti-apoptotic Bcl-xL and c-Fos were also up-regulated in the light. EPO-mediated neuroprotective effects attribute to interaction with Jak2, Bcl-xL, and c-Fos. Other downstream regulators such as Stat3 might be required for anti-apoptotic Bcl-xL induction as shown in the motor neuron [Schweizer et al, 2002]. Jak2 and Stat3 phosphorylations in retinal and RPE cells increased under hypoxia, hyperoxia, and light exposure.

Proangiogenic protein VEGF is up-regulated under hypoxic conditions in the retina and the RPE. Our data demonstrated that EPO and VEGF expressions are regulated in parallel, and they may interact directly or indirectly. We will test our hypothesis that pro-angiogenic signaling of VEGF regulated by EPO.

EPO and EPOR had a similar pattern of distribution in both neurons and astrocytes. Within 3 hrs after exposure to hypoxia, expression of EPO and EPOR in various retinal cells increased. The number of co-localized cells with EPO/EPOR with retinal cell markers also increased in 1% O_2 hypoxic condition. Relative levels of EPO mRNA were determined by real time-PCR in hypoxia. An elevated EPO level was detected after exposure of primary retinal cells to hypoxia (1% O_2) with 6-fold up-regulation after 12 h. We observed intense light induced up-regulation of c-Fos, Bcl-xL, thioredoxin pJak2, pSTAT3 in early passage of human RPE cells. Rat retina cells under bright light up-regulated pJak2, EPO, and EPOR in an hour. pJak2 and pStat3 in the RPE increased in hypoxic condition (1% O_2). Phosphorylation of Jak2 and Stat3 was confirmed as shown by phospho-Western blot. As an upstream regulator of EPO, HIF-1a was examined. HIF-1a increased in light, hyperoxic, and hypoxic conditions.

8. Cytoskeletal reorganization under oxidative stress

Not only intense light but also constant moderate light may trigger induction of anti-apoptotic Bcl-xL, EPO, and pro-apoptotic caspases. We postulate that light may induce post-translational modifications of target proteins. Previously, we identified that serine/threonine protein phosphatase 2A (PP2A) is induced and modified under constant light *in vivo*.

To our knowledge, protein nitration mechanism under various oxidative environmental conditions, including intense light and continuous light, has never been studied in retinal cell cultures, RPE cells, or mouse model system. Our contribution here is expected to be a detailed understanding of how PP2A modification of a subunit is regulated by endogenous signaling molecule, nitric oxide (NO). PP2A and vimentin might be critically involved in the process of light-induced retinal and RPE cell damage. It is expected that what is learned will be equally applicable to anti-apoptotic mechanism. In addition, the research will be of significance because what is learned will contribute to broader understanding of how other phosphatase activity can be modulated as an approach to therapy.

Although the potential importance of protein dephosphorylation as a therapeutic target has been appreciated, no detailed approach has been made targeting PP2A. Abnormalities in PP2A activity and concentrations have been linked to neurodegenerative diseases, including Alzheimer's and Parkinson's. The mechanism by which PP2A activity is regulated under oxidative stress remains largely elusive. Despite recent advances on structural investigation of PP2A, comprehensive understanding of dephosphorylation mechanism is far from complete.

Our data suggests that PP2A and vimentin are critically involved in the process of light-induced damage in the retina and RPE. By modulation of light-induced post-translational

modifications of PP2A, vimentin may assist in maintaining the proper filament network in Müller cells, which subsequently supports neuronal survival and architecture in the retina. Dephosphorylation switch by tyrosine nitration will provide new targets for therapeutic interactions in apoptosis-mediated disorders.

Light-induced photoreceptor damage depends on the functional visual cycle, a biochemical regeneration of 11-*cis*-retinal chromophore. The induction of light damage depends on the light condition; acute damage by intense light requires rhodopsin bleaching, whereas constant moderate light involves phototransduction. Recently we showed that the visual cycle is coordinated in a circadian manner as a means of effectively protecting the retina from the detrimental effects of light-induced, oxygen-dependent, free-radical-mediated damage, especially at the times of day when light is more intense [Chung et al, 2009]. RPE65 defects are known to trigger a remodeling of the retina that disrupt photoreceptor homeostasis and induce an apoptotic cascade causing retinal degeneration [Grimm et al., 2000; Wenzel et al., 2005]. As a downstream signal of light-induced signaling, anti-apoptotic proteins, including erythropoietin (EPO) and subsequent Janus kinase 2 (Jak2) phosphorylations, are tightly linked to a specific temporal period after oxidative stress and in anticipation of daily light onset. This is consistent with physiological protection against daily light-induced, oxidative mediated retinal apoptosis [Noell et al., 1966; Chung et al, 2009]. Continuous exposure to bright light (5000 lux) within 60 minutes induced apoptosis in retinal cells by activating caspase-3 signals. This suggests that endogenous neuroprotective proteins may not be sufficient in such intense oxidative stress conditions. Thus, we are seeking to define effective endogenous physiological neuroprotective strategies for anti-apoptosis from light-induced, oxygen-mediated damages that have clinical relevance.

The expression levels of a scaffold subunit of PP2Aa and vimentin are significantly increased when mice are exposed under continuous light for 7 days compared to control in rd 1 degeneration model [Zhang et al., 2010]. When melatonin is administered to animals while they are exposed to continuous light, the levels of vimentin and PP2A return to a normal level. Vimentin is a PP2A target of direct dephosphorylation. Vimentin is present in all mesenchymal cells and often used as a differentiation marker. Like other intermediate filaments, vimentin acts to maintain cellular integrity; further, vimentin may play a role in adhesion, migration, and cellular survival in the RPE. We demonstrated that bright light up-regulates pro-apoptotic signaling by cleavage of caspase-3 and Bcl-xL [Chung et al., 2009].

Exposure to continuous light at intensities ordinarily encountered during daytime causes retinal degeneration. How constant light induces retinal degeneration remains unknown, although potential mechanisms have been proposed, including defects in rhodopsin regeneration, accumulation of free radicals, and a continuous low level of Ca^{2+} resulting from an excessively active phototransduction cascade.

We examined the impact of constant light-exposed proteome changes *in vivo*. We exposed mice to either light (250-300 lux) for 12 hours followed by 12 hours of darkness or the same intensity of continuous light for seven days. Two proteins up-regulated by continuous light

were identified as PP2A and vimentin by mass spectrometry. We examined cytoskeletal proteins as potential substrates of PP2A. Cytoskeletal filament proteins were upregulated under constant light condition *in vivo*. Neurofilament, intermediate filament vimentin, tubulin, and actin increased in different levels under constant light exposure. The cytoskeletal target proteins were further analyzed by mass spectrometry to determine their modifications. Site-specific nitration and phosphorylations were found in PP2A and filament proteins.

Protein	Site	Modification
PP2Aa	Y169	nitration
Actin b cytoplasmic 1	T186	phosphorylation
Tubulin b 2B	Y50, Y51, T55	phosphorylation
Tubulin b 5	Y159	nitration
Tubulin b 5	S168, S172	phosphorylation
Vimentin	Y38, S325 ,S412, S420	phosphorylation

Table 3. Modifications of PP2A and filament proteins under constant light *in vivo*

Vimentin is a major component of intermediate filament that spread throughout the cell and serve as signal transducer conveying mechanical and molecular information from cell surface to nucleus and intracellular compartments. Neuronal cytoskeleton is a potential therapeutic target in neurodegenerative diseases. Vimentin phosphorylations were further analyzed by 2D electrophoresis with narrow range of pI for higher resolution. Vimentin phosphorylation decreased under constant light *in vivo*. Modifications of vimentin are essential reactions of filament dynamics. Changes of vimentin phosphorylation are directed to reorganization of the intermediate filament network and altered function of Müller cells.

Hyperphosphorylation of vimentin can disassemble intermediate filament into soluble monomeric form. Müller cells are the major glial cells in the retina and play crucial roles in maintaining neuroretinal architecture and support neuronal survival. Our results suggest that vimentin and Müller cells might be critically involved in the process of light-induced damage in the retina. By reducing the light-induced post-translational modification of vimentin, PP2A may assist to maintain the proper filament network in Müller cells, which subsequently supports neuronal survival and architecture in the retina. To test changes of PP2A and cytoskeletal proteins in RPE *in vitro*, we examined ARPE-19 cells under stress condition. When RPE cells were under oxidative stress (100-250 μM H_2O_2), a catalytic subunit PP2Ac was initially upregulated, then decreased in time and dose dependent manner.

A decrease in cytoskeletal protein turnover leads to the accumulation of large aggregates of actin and tubulin, which triggers an increase in the levels of reactive oxygen species (ROS). Dynamic and differential changes in the cytoskeleton occur in apoptotic cellular processes. Thus, we ask how phosphorylation of vimentin regulates cytoskeletal dynamics and cell survival under stress conditions in RPE. We hypothesize that the modifications of PP2A and vimentin influence the cytoskeletal network through interplay with other cytoskeletal proteins. A stabilized vimentin may act as an antiapoptotic agent when cells are under stress.

Methylation and phosphorylation of PP2A catalytic c subunit are evolutionary conserved mechanisms that critically control the PP2A holoenzyme assembly and substrate specificity. However, interplay of PP2A nitration and phosphorylation that may control subunit binding has never been studied.

Regulation of tyrosine nitration could be a potential intervention of early stage light-induced ocular diseases. We postulate, on the basis of our data, that modifications of PP2A and vimentin influence the cytoskeletal network through interplay with other cytoskeletal proteins, including tubulin and actin. A positive correlation between the levels of PP2A and vimentin under light-induced stress suggests that cytoskeletal dynamics is regulated by dephosphorylation of vimentin as a PP2A substrate.

To examine PP2A and cytoskeletal proteins under oxidative stress, we examined light-induced cytoskeletal changes using ARPE-19 cells. ARPE-19 contains retinal G-protein receptors (RGR) and peropsin as light detecting chromophores. The assembly of the major cytoskeleton proteins, including vimentin, tubulin and actin, are highly interdependent. Thus, we investigated β-tubulin and the interplay of vimentin dynamics with microtubules under light. Tubulin is a highly concentrated building block (10–20 μM) of microtubules. β-tubulin is aggregated as shown by particles (white arrow) under intense light (Figure 5). b-tubulin is colocalized with mitochondria in dark, but changed localization under light condition. Confluent ARPE-19 cells were treated with either dark or 7000 lux white light for 2 hr. Mitochondria was stained by 100 nM MitoTracker Orange. β-tubulin was visualized by Alexa 488 secondary antibody and the nucleus was stained by DAPI. To follow vimentin-tubulin interaction changes in light, we analyzed tubulin polymerization rates and vimentin-tubulin colocalization. β-tubulin is a major component of the microtubule and also an essential component of the cytoskeleton. They play a critical role in cell division and cell motility. Our results demonstrated that actin filament also aggregated in cytosol under intense light condition in RPE. In the dark, β-actin is localized in cytosol, but in light β-actin is aggregated outside of mitochondria. Light-induced modification of cytoskeletal proteins may imply a potential regulatory mechanism in apoptosis. Direct interaction between vimentin and actin has been observed [Esue et al., 2006]. The tail domain of vimentin intermediate filaments interacts directly with actin, which may regulate cytoskeletal crosstalk. Morphologic change and cytoskeletal reorganization of RPE cells were observed under oxidative stress *in vitro*.

9. The visual cycle

The steady-state of retinoid concentration and photosensitivity are controlled by the biosynthetic pathway leading to 11-*cis*-retinal regeneration, which is called the visual cycle. For continued vision, 11-*cis*-retinoid must be regenerated by a series of enzymes, including retinol dehydrogenases (RDHs), lecithin retinol acyltransferase (LRAT), and RPE65 [Saari 2000; Rando 2001; Jahng et al 2002; Jahng et al., 2003a; Jahng 2003b; Bok et al., 2003; Xue et al., 2004; Xue et al., 2006] (Figure 1).

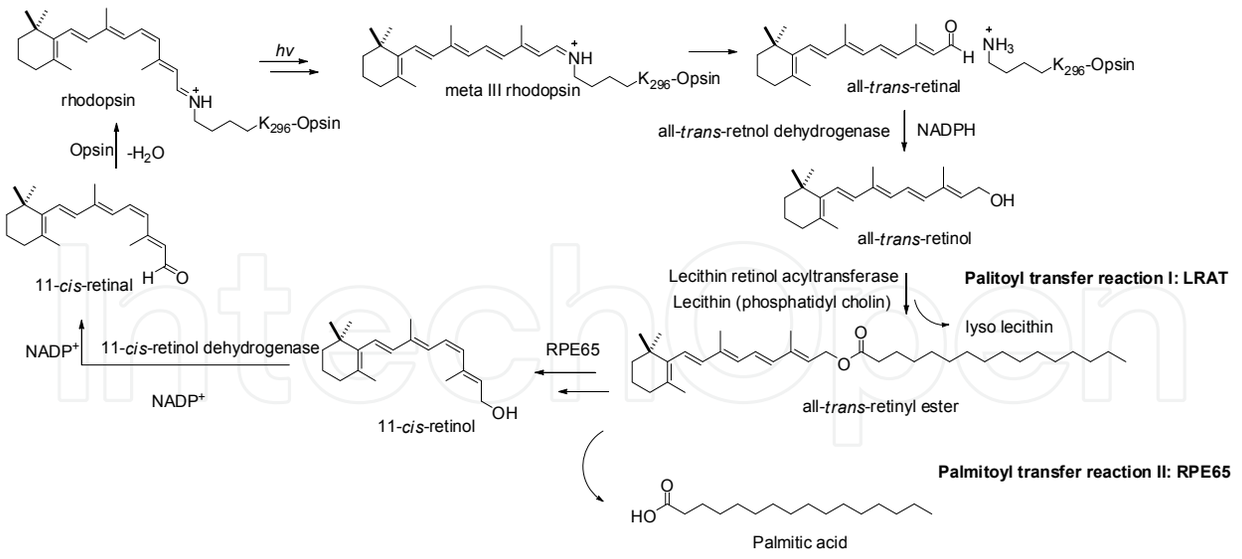

Figure 1. Biochemical reactions in the visual cycle and two palmitoylation transfer steps.

RPE is susceptible to oxidative stress due to its high oxygen consumption, the generation of reactive oxygen species (ROS), the presence of a high percentage of unsaturated fatty acids, and exposure to light [Bok, 1993; Strauss, 2005]. RPE65, a peripheral membrane protein of RPE cells, is thought to be an all-*trans*-retinyl ester isomerohydrolase [Moiseyev et al, 2005; Jin et al, 2005; Redmond et al, 2005]. In conjunction with other proteins, it isomerizes all-*trans*-retinyl ester and hydrolyzes it into 11-*cis*-retinol, the immediate precursor to 11-*cis*-retinal Schiff base chromophore which is enzymatically regenerated in the RPE. RPE65 was originally identified as the putative RPE membrane receptor for the plasma retinol-binding protein, and later shown to be a retinoid binding protein with high affinity toward all-*trans*-retinyl palmitate [Hooks et al, 1989; Bavik et al, 1991; Bavik et al, 1992; Bavik et al, 1993; Hamel et al, 1993a; Hamel et al, 1993b; Tsilou et al, 1997; Ma et al., 2001; Jahng et al., 2003a; Gollapalli et al, 2003; Mata et al, 2004; Xue et al, 2004]. It is homologous to beta-carotene monooxygenase [Kloer et al., 2005].

RPE contains anti-apoptotic and neuroprotective factors to support retina survival and maintenance. With aging, an imbalance occurs between ROS production and the capacity for detoxification resulting in the accumulation of ROS and the diminution of mitochondrial respiratory function. These ROS may contribute to the development of eye diseases such as cataract [Spector, 1995], uveitis [Satici et al, 2003; Bosch-Morell et al, 2002], glaucoma [Osborne et al, 1999; Babizhayev et al, 1989], retinopathy of prematurity [Dani et al, 2004; Head 1999], AMD [Beatty et al, 2000; Spraul 1996], and diabetic retinopathy [Kowluru et al, 1994]. Thus, understanding the mechanism of elevated ROS during aerobic metabolism and the resulting oxidative stress is important to the treatment of eye diseases. Under sub-lethal conditions of oxidative stress, mitochondrial respiratory function is impaired by electron leakage [Yakes and Van Houten, 1997; Melov et al, 1999] leading to decreased ATP production. Moreover, elevated ROS induce mutations in mitochondrial DNA, oxidative damage to cellular macromolecules forming cross-linked aggregates, and accumulation of damaged proteins [Grune et al, 1997].

Several mutations in the *RPE65* gene have been shown to be associated with retinal degenerations, including autosomal recessive childhood-onset severe retinal dystrophy, Leber's congenital amaurosis (LCA), and retinitis pigmentosa [Gu et al., 1997; Chen et al., 2006; Cottet et al., 2006; Hamel et al., 2001; Travis et al., 2007; Felius et al., 2002]. The RPE65 L450M mutant is associated with the reduced concentration of A2E lipofuscin [Kim et al., 2004]. RPE65 is essential for 11-*cis*-retinol biosynthesis *in vivo* and *in vitro* [Redmond et al., 1998; Nicolaeva et al., 2009]. Studies using RPE65 knockout mice demonstrate the importance of RPE65 in 11-*cis*-retinal biosynthesis *in vivo* [Redmond et al, 1998]. The synthesis of 11-*cis*-retinal is eliminated in these mice, while all-*trans*-retinyl esters accumulate as oil droplets in RPE cells. The control elements that regulate the overall biosynthetic pathway to 11-*cis*-retinal under light and oxidative stress are not known. Furthermore, connection between the visual cycle and resulting redox imbalance is in question.

In 1989, Rando proposed that RPE membranes might be the energy source for isomerization of all-*trans*-retinyl ester to 11-*cis*-retinol [Deigner et al., 1989]. In his hypothesis, the free energy of hydrolysis of the retinyl ester is coupled to the endothermic *trans* to *cis* isomerization. However, there are series of questions inlcuding; 1) C11 position is not nucleophilic and palmitate is not a good leaving group; 2) isomerization of the conjugated double bond of the retinoid cannot be explained in this mechanism. In 1999, Palczewsky proposed a carbocation intermediate mechanism [Stecher et al., 1999]. They suggested that the regeneration of 11-*cis* retinal might occur through a retinyl carbocation based on the observation of inhibition by positively charged retinoid as transition state analog. Both hypotheses do not explain the 11-*cis*-specific isomerization reaction of conjugated double bonds. Rather, singlet oxygen may bind to iron (Fe^{2+}) in the active site of RPE65 and catalyze C11 specific isomerization through oxygen radical-mediated epoxide intermediate as shown in Figure 2.

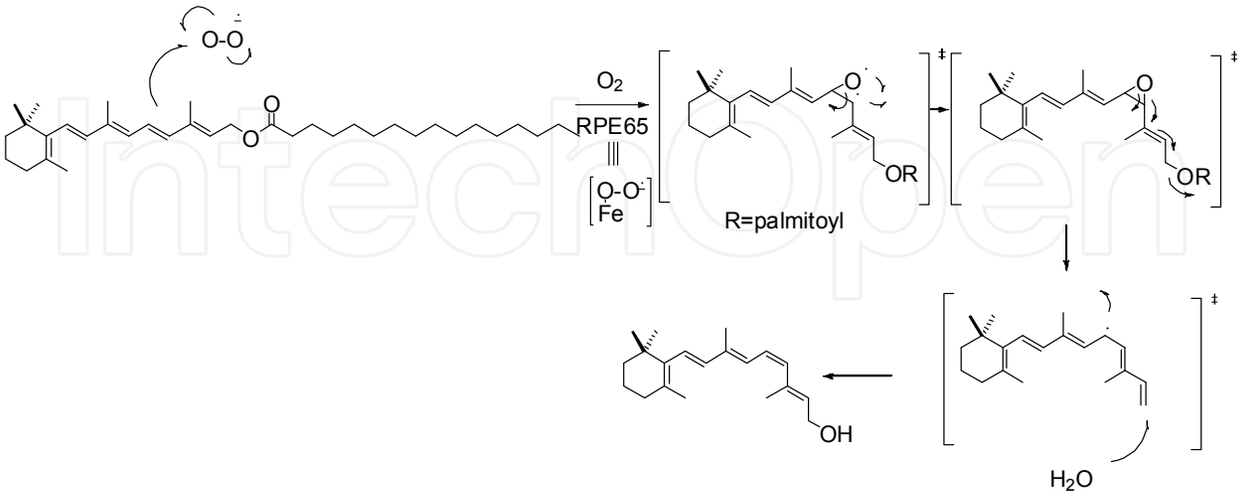

Figure 2. Putative Retinoid Isomerization Mechanism

Recently, we found that levels of RPE65 and RPE45, as a truncated form of RPE65, increased after short-term exposure to intense or constant light in the human RPE cultures [Chung et

al., 2009; Lee et al., 2010a]. Also, the level of RPE45 is present in cells exposed to high oxidative stress *in vitro* and *in vivo* [Lee et al., 2010a]. We further suggest that the RPE45 fragment may be generated via ubiquitination involving interaction of specific proteases with RPE65. We also identified that retinaldehyde binding protein (CRALBP1) in the Müller cell showed a reversal of expression under constant light after melatonin treatment compared to constant light only condition (light vs. light+melatonin) [Zhang et al., 2010). Light-induced CRALBP1 may accelerate the retinal damage through higher rate of the visual cycle.

Interestingly, C57BL/6 mice have a gene polymorphism (M450) associated with lower level of RPE65 expression and reduced light-damage sensitivity. We observed a higher threshold for RPE45 appearance when RPE cells were exposed to light and oxidative stress simultaneously compared to cells under oxidative stress alone, suggesting that the protective mechanism may exist to maintain RPE65 concentration under light-induced oxidative stress.

We examined the appearance of RPE45 and RPE20 fragments *in vitro* with different amounts of H_2O_2 (100, 300, 500 and 1000 μM) in order to mimic different levels of oxidative stress on bovine RPE cells. As expected, RPE45 and RPE20 were produced in a dose-dependent manner after H_2O_2 treatment.

To address the functional significance of RPE45, we tested the ability of the RPE45 fragment to bind biotinylated all-*trans*-retinyl chloroacetate (BRCA), an all-*trans* retinyl ester analog used to investigate the retinoid-binding roles of RPE proteins. In this experiment, RPE proteins from bovine RPE cells were incubated with BRCA 50 μM/100 mg RPE proteins and separated by SDS-PAGE. BRCA-labeled proteins were visualized using an avidin-peroxidase antibody and an anti-RPE65 peptide antibody. BRCA-labeled RPE65, RPE45 and LRAT were confirmed by electrospray tandem mass spectrometry and Western-blot analyses, demonstrating that RPE45 binds this all-*trans*-retinyl ester analog.

A defect in RPE65 can trigger a remodeling of the retina that may disrupt photoreceptor homeostasis and induce apoptosis cascades leading to retinal degeneration [Shang and Taylor, 2004]. Light influences the translocation of transducin, arrestin, and recoverin; tyrosinase-mediated light adaptation, and the synthesis of retinoic acid and melatonin. Our studies indicate that oxidative stress and light exposure can influence the visual cycle. The dose-dependent induction of RPE45, a fragment of RPE65 generated in response to H_2O_2 or light exposure by a specific caspase-mediated cleavage suggests that RPE45 is a potential signaling molecule of oxidative stress- and light-induced apoptosis [Chung et al., 2009; Lee et al., 2010a]. Proteolysis after stress is an important signal transduction pathway mediated by caspases, presenilin, and amyloid precursor protein, which can lead to neurodegenerative disorder or inflammation [Martinon and Tschopp 2004; Shi 2004; Haass and Strooper 1999]. We also observed up-regulation of NF-kB and amyloid beta in the RPE under oxidative stress. RPE65 is known as highly uveitogenic and antigenic protein implying involvement in pathogenic autoimmune diseases in the eye [Ham et al, 2002]. Changes in RPE65 expression are also seen *in vivo* during cycles of light and dark and may be mediated by light cues.

In nature, all biochemical reactions are dependent on the coupling of the free energy changes of phosphate- (ATP), thio- (CoA), and oxy- (membrane lipids) esters. For all-*trans*-retinyl ester isomerization/hydrolysis biochemical reactions catalyzed by RPE65, palmitoyl transfer reaction should be coupled with retinyl ester isomerization and retinyl ester hydrolysis energetically and chemically.

We suggest that RPE45 may result from degradation of RPE65 by ROS-induced proteases [Budihardjo et al., 1999; Santoro et al., 1998]. It is likely that RPE cells have a defense mechanism against oxidative stress and we therefore investigated the expression of anti-apoptotic factors in oxidative stress. We found that anti-apoptotic Bcl-xL was increased under intense light [Chung et al., 2009]. The positive correlation between the expression of these anti-apoptotic factors and RPE65 under oxidative stress suggests that RPE65 may have a positive role in apoptotic signaling. Proteolysis after stress is an important signal transduction mediated by caspases, presenilin, and amyloid precursor protein, which can lead to neurological disorder or inflammation in the retina and brain [Martinon and Tschopp 2004, Shi 2004, Haass and Strooper 1999]. RPE65 is known as highly uveitogenic protein implying involvement in pathogenic autoimmune disease in the eye [Ham et al. 2002]. Under oxidative stress, aged cells increase the number of mitochondria and induce anti-oxidant genes [Sitte et al, 2000; Lee et al, 2000]. Retinoid map in the retina and the RPE is shown in Figure 3. Potential target sites to regulate retinoid and A2E are shown in red bars.

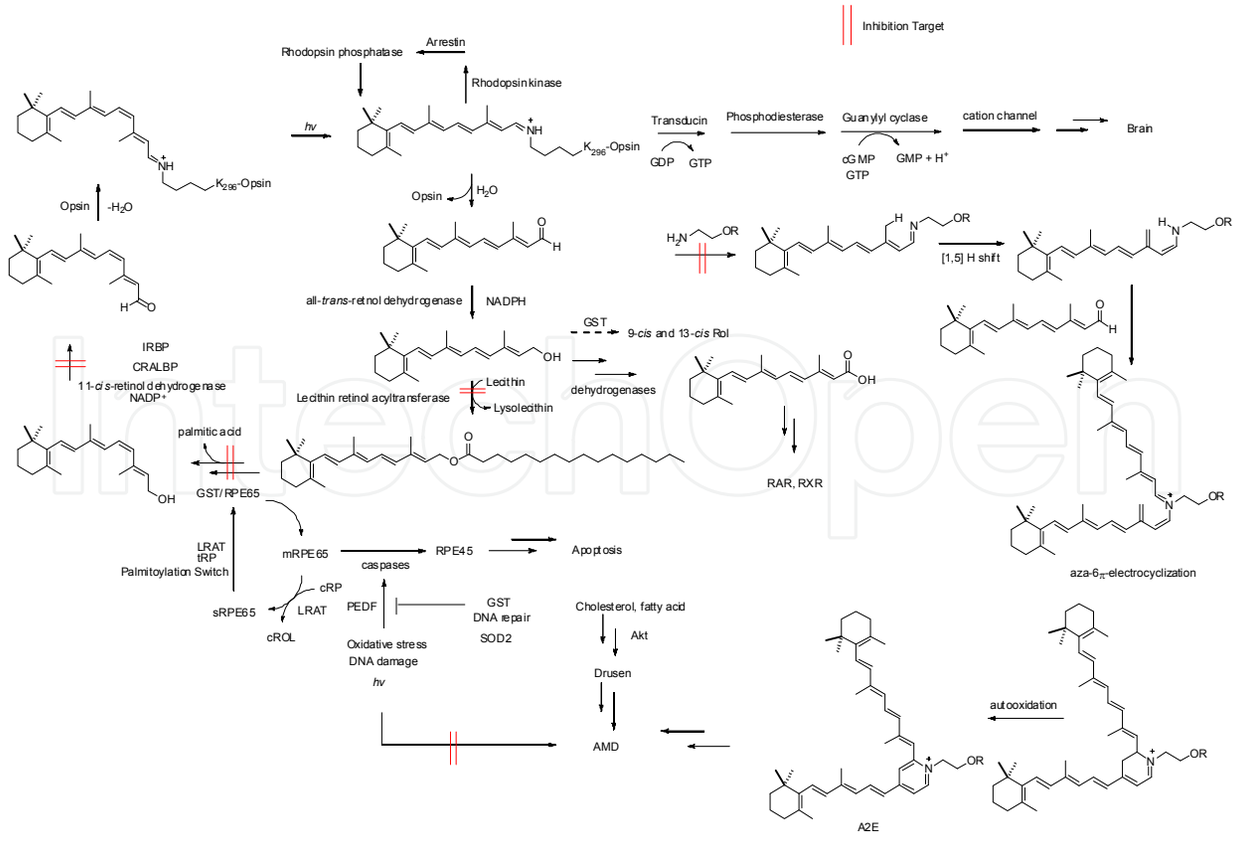

Figure 3. Retinoid Map in the Retina and RPE cells

10. Conclusion

While the end point of apoptosis is well established, there is still a large gap between knowledge of early biochemical events and the end stage of age-related macular degeneration (AMD). Proteome changes under oxidative stress have been studied in regard to the pathogenesis of AMD [Yang et al 2006]. Understanding the molecular mechanism of proteomic signaling will provide a novel insight into apoptotic processes in AMD. It is expected that the knowledge will be equally applicable for understanding the lipid-mediated cell death mechanism.

Light-induced retinal degeneration in animal model occurs only when the visual cycle is functional. Regeneration of 11-*cis*-retinal as a chromophore of rhodopsin is depending on biochemical reactions of retinoid processing enzymes, including lecithin retinol acyltransferase and RPE65 [Xue et al., 2004; Xue et al., 2006; Jahng et al., 2003a; Jahng et al., 2003b; Jahng et al., 2002; Bok et al., 2003]. Activity and expressions of these enzymes might be controlled by circadian regulators or daily light onset [Xue et al., 2004; Chung et al., 2009; Lee et al., 2010a]. A study of RPE65 knockout mice exhibited that light damage only occurs when the retina is supplied with 11-*cis* retinal [Wenzel et al., 2005]. Additional evidence in RPE65 L450M mice showing slow rhodopsin regeneration, halothane anesthesia as inhibition method of 11-*cis*-retinal regeneration, and 13-*cis*-retinoic acid as a putative RPE65 inhibitor imply that continuous regeneration of 11-*cis*-retinal is one of the key steps to induce retina degeneration [Wenzel et al., 2005]. Our goal is to explicate the role of light and time under oxidative stress in the control of neuroprotective protein expressions in the retina and the RPE [Zimmermann et al., 2006]. Our questions include whether antiapoptotic factors, that include prohibitin, nitric oxide, vimentin, PP2A, and erythropoietin can protect retina and RPE cells against oxidative- or light-induced apoptotic neurodegeneration at specific time points.

Our proteomic approaches to understand RPE cell death under stress conditions demonstrate that: 1) crystallins are upregulated and hyperphosphorylated. 2) neuroprotective erythropoietin and subsequent JAK2 phosphorylations are tightly linked to a specific time after oxidative stress and in anticipation of daily light onset. 3) early signaling molecules, including mitochondrial prohibitin, changes their expression, subcellular localization, phosphorylation, and lipid interaction under oxidative stress. 4) relative lipid compositions, including phosphatidylcholine and cholesterol, are altered under oxidative stress. 5) oxidative stress leads to cytoskeletal reorganization through site-specific vimentin phosphorylations that regulate intermediate filaments, resulting in nonfilamentous particles.

AMD is characterized in its early stages by the presence of extracellular deposits, known as drusen, that accumulates between the basal surface of the RPE and Bruch's membrane. During the past decade, compelling evidence has emerged implicating the immune system and the complement system in particular in drusen biogenesis and AMD. A number of the proteins detected in drusen are either complement components or related molecules. Despite these significant advances, the identity of the molecules responsible for triggering activation of the complement cascade, as well as the downstream molecular interactions that

promote AMD pathology, remain elusive. Our proteomics studies will provide new insight into the underlying mechanisms involved in the development and progression of AMD and further elucidate the relationship between various risk facotors, including oxidative stress and complemnt activation. Such information are critical for the development of more effective therapeutic strategies for the treatment of retinal degeneration that includes AMD.

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