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Interactome Mapping Guided by Tissue-Specific Phosphorylation in Age-Related Macular Degeneration

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Abstract

The current study aims to determine the molecular mechanisms of age-related macular degeneration (AMD) using the phosphorylation network. Specifically, we examined novel biomarkers for oxidative stress by protein interaction mapping using *in vitro* and *in vivo* models that mimic the complex and progressive characteristics of AMD. We hypothesized that the early apoptotic reactions could be initiated by protein phosphorylation in region-dependent (peripheral retina vs. macular) and tissue-dependent (retinal pigment epithelium vs. retina) manner under chronic oxidative stress. The analysis of protein interactome and oxidative biomarkers showed the presence of tissue- and region-specific post-translational mechanisms that contribute to AMD progression and suggested new therapeutic targets that include ubiquitin, erythropoietin,

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COMPETING INTERESTS

None

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vitronectin, MMP2, crystalline, nitric oxide, and prohibitin. Phosphorylation of specific target proteins in RPE cells is a central regulatory mechanism as a survival tool under chronic oxidative imbalance. The current interactome map demonstrates a positive correlation between oxidative stress-mediated phosphorylation and AMD progression and provides a basis for understanding oxidative stress-induced cytoskeletal changes and the mechanism of aggregate formation induced by protein phosphorylation. This information could provide an effective therapeutic approach to treat age-related neurodegeneration.

Keywords

AMD; retinal pigment epithelium; oxidative stress; protein interactome; mitochondria; proteomics

Introduction

In an oxidative environment, eukaryotic cells exhibit central signaling responses including ATP-mediated reversible protein phosphorylation at serine, threonine, or tyrosine residues [1,2]. These transient post-translational modifications in response to extracellular and mitochondrial stressors control apoptosis of retinal pigment epithelial (RPE) cells within a very short timeframe. The RPE is a supportive layer of cells that are essential for the health of rod and cone photoreceptors [2]. Progressive cell death of post-mitotic RPE can lead to age-related macular degeneration (AMD), which results from rod and cone apoptosis. RPE cells are exposed to chronic oxidative stress, including constant exposure to intense light and reactive oxygen species (ROS) from mitochondria due to high levels of oxygen consumption. However, the mechanisms of oxidative stress-mediated phosphoproteomic alterations that initiate death signals in the RPE remain elusive. Further, details regarding phosphorylation signaling in AMD are complicated due to the dynamics of phosphorylation reactions lasting from seconds to minutes, as compared to the slow kinetics of the aging process, which occurs over years in the retina.

Proteomic approaches that include 2D gel electrophoresis and protein identification by mass spectrometry have been used routinely for the last decade to study protein identification and modifications. However, RPE phosphoproteomics in AMD remains challenging due to the complex metabolism and sub-stoichiometry of phosphoproteins. For this reason, proteomic studies using *in vivo* models that mimic the complex and progressive characteristics of AMD are extremely valuable for studying the pathogenesis of AMD and testing different treatment modalities [3–6].

Unbiased proteomic approaches demonstrated that the early apoptotic network and post-translational mechanisms exist to determine RPE cell survival or death under stress conditions. Early signaling molecules under oxidative stress, including erythropoietin (EPO), crystallins, vimentin, protein phosphatase 2A (PP2A), hypoxia inducible factor-1 (HIF1), JAK2, prohibitin, RPE65, and nitric oxide synthase (NOS), are modulated and hyperphosphorylated *in vitro* and *in vivo* [7–9]. For instance, phosphorylations of crystallin and vimentin may participate in the pathogenesis of AMD by forming soft drusen with longer chain phosphatidylcholine and cholesteryl esters [10,11].

In the current study, we seek to determine whether tightly regulated phosphorylation reactions in RPE cells are critical to maintain cytoskeletal integrity and cell survival under chronic oxidative stress. Our objective is to examine the molecular network of altered phosphorylation to determine initial targets to treat AMD in the early stage. We examined a novel role of phosphorylated biomarkers as an adaptation to extracellular stress and used the phosphoproteome interaction map to determine the early molecular mechanisms of RPE apoptosis in AMD.

We have built a comprehensive interaction map by combining several independent sets of *in vivo* and *in vitro* data including immunoprecipitation, co-expression, protein domain, and published data from the literature. A large scale phosphorylation analysis demonstrated that multiple phosphorylation motifs are implicated in the mechanisms of AMD. A combination of phosphopeptide enrichment, high performance liquid chromatography, and electrospray tandem mass spectrometry, followed by database search, provides an integrated phosphoproteome showing the apoptotic pathway, energy metabolism, inflammation, cytoskeletal rearrangement, and mitochondrial network in AMD.

Materials and Methods

We followed the NIH Guide and the Association for Research in Vision and Ophthalmology (ARVO) statement for *in vivo* experiments. The animal protocol was approved by the Institutional Animal Care and Use Committee.

Sprague-Dawley (SD) rats (male, 250–300 g) and mice (C57/BL6J genetic background) were purchased from Charles River Laboratories (Wilmington, MA). As a positive control for oxidative stress in the retina, diabetic condition was induced by intravenous injection of streptozotocin (65 mg/kg in 0.1M sodium citrate, pH 4.5, Sigma-Aldrich, St. Louis, MO). Negative control animals received injections of vehicle alone. Rats and mice (n=9, biological triplicate and technical triplicate) were considered to be diabetic at blood sugar levels >350 mg/dL. Animals were euthanized 2 or 4 weeks following onset of diabetes by an overdose of anesthetic. Comparative analysis was conducted with an identical animal strain (SD). Control SD rats and mice were sacrificed at 12 weeks of age and aged SD rats and mice were sacrificed at 52 and 54 weeks of age. Retinas from rats and mice were removed and frozen in liquid nitrogen. Retinas from different subgroups were collected and prepared for biochemical analysis. All experiments were repeated (n=3 biological samples) with technical triplicate (n=9).

Stat View software was used for statistical analysis. Statistical significance was analyzed using unpaired Students' test or variance (ANOVA) when appropriate. The significance level $P < 0.05$ is considered as statistically significant.

Donor Eye Tissue and Phosphoprotein Enrichment

Human postmortem donor eye tissues were used following the tenets of the Declaration of Helsinki. Diabetic retinopathy (DR) human retinal tissues (n=9, biological triplicate $\times$ technical triplicate) were obtained from the Georgia Eye Bank (Atlanta, GA,). Human age-related macular degeneration (AMD) retina (8 mm macular and peripheral punches), RPE (8

mm central and peripheral punches), and age-matched control eyes (n=9, biological triplicate $\times$ technical triplicate) were provided by the Lions Eye Bank (Moran Eye Center, University of Utah). Phosphoproteome of macular (I), peripheral retina (II), central RPE (III), and peripheral RPE (IV) were compared to age-matched control donor eyes to determine region-specific, senescence-associated molecular mechanisms during AMD progression. Phosphoproteins were enriched by charge-based spin column chromatography and resolved by 2D gel electrophoresis as previously reported [12]. In addition, trypsin digested phosphopeptides from whole lysates were enriched using $\text{Ga}^{3+}/\text{TiO}_2$ immobilized metal ion chromatography. Eluted phosphopeptides were analyzed using MALDI-TOF-TOF and ESI MS/MS. Serine, threonine, and tyrosine phosphorylations were confirmed by phospho-Western blotting analysis (data not shown).

ARPE-19 and HRP Cells

For *in vitro* experiments, retinal pigment epithelial cells (ARPE-19) were purchased from ATCC (Manassas, VA) and retinal progenitor cells (HRP) were kindly donated by Dr. Harold J. Sheeldo at the University of North Texas Health Science Center. ARPE-19 and HRP cells were cultured in a 5% CO_2 incubator at 37°C in 100 mm dishes (Nalge Nunc International, Naperville, IL) using Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (10%) and penicillin/streptomycin (1%). Confluent cells were trypsinized (5–7 minutes at 37 °C) using a trypsin-EDTA buffer (0.1%, Sigma-Aldrich, St. Louis, MO), followed by centrifugation (300 $\times g$, 7 min). Cells (eight to nine passages) were grown to confluence for 2–4 days and then were treated with H_2O_2 (200 μM), intense light (7,000–10,000 lux, 1–24 hrs) or constant light (700 lux, 48 hrs). HRP and ARPE-19 cells were rinsed (Modified Dulbecco's PBS) and lysed using IP lysis buffer containing Tris (25 mM), NaCl (150 mM), EDTA (1 mM), NP-40 (1%), glycerol (5%), and protease inhibitor cocktail at pH 7.4 by incubating on ice for 5 minutes with periodic sonication (3 $\times$ 5 min), followed by centrifugation (13,000 $\times g$, 10 minutes). Proteins (1 mg/ml, 200–400 μL) were loaded for immunoprecipitation and nonspecific bindings were avoided using control agarose resin cross-linked by 4% bead agarose. Amino-linked protein-A beads were used to immobilize antiprohibitin antibody with a coupling buffer (1 mM sodium phosphate, 150 mM NaCl, pH 7.2), followed by incubation (room temperature, 2 hrs) with sodium cyanoborohydride (3 μL , 5 M). Columns were washed using a washing buffer (1 M NaCl), and protein lysate was incubated in the protein A-antibody column with gentle rocking overnight at 4 °C. The unbound proteins were spun down as flowthrough, and the columns were washed three times using washing buffer to remove nonspecific binding proteins. The interacting proteins were eluted by incubating with elution buffer for 5 minutes at RT. The eluted proteins were equilibrated with Laemmli sample buffer (5 $\times$, 5% β -mercaptoethanol). Eluted proteins were separated using SDS-PAGE and stained using Coomassie blue (Pierce, IL) or silver staining kit (Bio-Rad, Hercules, CA). Immunoprecipitated proteins were reported previously [13] and were used to establish interactome in the current study.

Two dimensional SDS-PAGE

Protein samples were purified by ReadyPrep 2-D Cleanup Kit (Bio-Rad, CA) and quantified using BCA protein assay kit (Pierce, Rockford, IL). 150 μg of protein was incubated in 200 μL of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS and 50 mM DTT)

supplemented with 0.5% destreak IPG buffer, pH 3–10 (GE healthcare, PA). Isoelectric focusing was carried out using 11 cm immobilized dry strips (Bio-Rad, CA) with a linear pH 3–10 and 4–7 gradients. Strips were rehydrated for 12 hrs at 20 °C. Proteins were separated by the Ettan IPGphor-3 (GE healthcare) using programmed voltage gradients at 20 °C for a total of 12 kVh (1 h at 500 V, 1 h at 1000 V, 2 hrs at 6000 V and 40 min at 6000 V). The IPG strips were reduced in equilibration buffer I (0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 50 mM DTT) for 20 min at 25 °C and alkylated for 20 min in equilibration buffer II containing 150 mM iodoacetamide. The equilibrated strips were placed on top of polyacrylamide gels (8–16%, Bio-Rad, CA) and sealed with 1% agarose buffer. Proteins were visualized by Coomassie staining using Imperial Protein Stain (Pierce, Rockford, IL) and Western blot.

Mass Spectrometry Analysis

Protein bands were excised (1×1×1 mm). The Coomassie-stained or silver-stained gel pieces were incubated using a Coomassie destaining buffer (200 μ L of 50% MeCN in 25 mM NH_4HCO_3 , pH 8.0, room temperature, 20 minutes) or silver destaining buffer (50% of 30 mM potassium ferricyanide, 50% of 100 mM sodium thiosulfate, 5–10 minutes). The gel pieces were dehydrated (200 μ L, MeCN) and vacuum-dried (Speed Vac, Savant, Holbrook, NY). Disulfide bonds were reduced by DTT (10 mM) in the NH_4HCO_3 buffer (100 mM) for 30 minutes at 56 °C, and then were alkylated by iodoacetamide (55 mM) in the NH_4HCO_3 buffer (100 mM) for 20 minutes at room temperature in the dark. Proteins were digested using trypsin (13 ng/ μ L sequencing-grade from Promega, 37 °C, overnight) in NH_4HCO_3 (10 mM) containing MeCN (10%). The peptides were enriched using a buffer (50 μ L of 50% MeCN in NH_4HCO_3 , 5% formic acid, 20 minutes, 37 °C). Dried peptides were dissolved in a mass spectrometry sample buffer (5–10 μ L, 75% MeCN in NH_4HCO_3 , 1% trifluoroacetic acid). Alpha-cyano-4-hydroxycinnamic acid (5 mg/mL, MW 189.04, Sigma-Aldrich, St. Louis, MO) was freshly dissolved in a matrix buffer (50% MeCN, 50% NH_4HCO_3 , 1% trifluoroacetic acid) and centrifuged (13,000 $\times g$, 5 minutes). The peptide-matrix mixtures (0.5 μ L) were spotted onto the MALDI Plate (Ground steel, Bruker Daltonics, Germany). Mass spectrometer and all spectra were calibrated using the known peptide, trypsin (842.5099 Da, 2211.105 Da). The mass spectrum was recorded in 800–3000 Da range using Flex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany, 70–75% laser intensity, 100–300 shots). Mass spectrometry data were analyzed using Flex analysis software (Bruker Daltonics, Germany). Peptides were analyzed using the Mascot software (Matrix Science) and NCBI/SwissProt database (zero mismatch cleavage, carbamidomethyl cysteine, methionine oxidation, 50–300 ppm mass tolerance, S/T/Y phosphorylation = 79 m/z search). Peptide identification was evaluated based on Mascot MOWSE score, number of matched peptides, and protein sequence coverage. MOWSE score is expressed as $-10\log P$ as a probability value to compute the composite probability P. The experimental details for the ESI MS/MS experiments were reported previously [14–16].

Prohibitin Interactome Map

AMD and oxidative biomarkers interactome were established using protein-protein interaction map software and databases, including STRING 10.0 (<http://string-db.org/>), MIPS (<http://mips.helmholtz-muenchen.de/proj/ppi/>) and iHOP (<http://www.ihop-net.org/>)

UniPub/iHOP/). Proteins found in AMD or oxidative stress conditions were added to establish the AMD interactome. Protein interactions were presented using eight categories, including neighborhood (green), gene fusion (red), co-occurrence (dark blue), co-expression (black), binding experiments (purple), databases (blue), text mining (lime), and homology (cyan). Protein interactions were determined and confirmed by genomic context, high-throughput experiments, co-expression, and previous publications in Pubmed. Protein database analysis showed the region-specific phosphorylation of specific proteins in AMD eyes. The interactome between AMD proteome was compared to the retina/RPE proteome under stress conditions.

Lipid analysis

Lipids were extracted from ARPE-19 cells using chloroform-methanol (2:1, v/v) and organic solvent was evaporated under a gentle nitrogen stream and dissolved in chloroform for analysis by HPLC and mass spectrometry.

RESULTS

Phosphoproteome Changes in AMD

To determine whether early biomarkers in the phosphoproteome may play crucial roles in the progression of apoptotic signaling in AMD, we introduced a system-wide, unbiased, and high-throughput approach to examine the global phosphoproteome and the AMD interactome using a phosphoproteome enrichment method and protein interaction mapping.

The phosphoproteome of macula (I), peripheral retina (II), central RPE (III), and peripheral RPE (IV) in AMD donor eyes were compared to age-matched control donor eyes to determine region-specific, senescence-associated protein phosphorylation during AMD progression (Figure 1). Phosphoproteins from four different tissues were collected by using Ga^{3+} -based column chromatography and analyzed by two-dimensional gel electrophoresis. Phosphoproteins were identified by trypsin digestion, followed by MALDI-TOF-TOF mass spectrometry analysis. Altered phosphoproteins from peripheral regions of the neural retina (II) in AMD eyes were identified as follows; transferrin, pyruvate kinase, enolase, unnamed protein ($\alpha 2$ macroglobulin MG1 domain), PAK S/T kinase, protein phosphatase (PP2A), GFAP, tyrosine protein kinase FES/FPS, dynamin-like protein, creatine kinase, and vimentin (Figure 2A, Table 1). The presence of these phosphoproteins implies that iron homeostasis, energy regeneration, glycolytic flux, and cytoskeletal structure could be affected during AMD progression. In contrast, altered phosphoproteins from peripheral RPE (IV) were ubiquinol oxidoreductase, inositol 1,4,5 triphosphate receptor, calponin, ankyrin repeat domain 30B, guanylate cyclase, sorting nexin, and coiled-coil domain protein 73. The above phosphoproteins suggest potential alterations of fast energy transition, oxidative damage, blood flow, physiological hypoxia, cGMP, and apical polarity in AMD (Figure 2B, Table 1).

Phosphopeptides were obtained by trypsin digestion, followed by enrichment protocol by using Ga^{3+} -immobilized ion chromatography to confirm specific phosphoproteins. Eluted phosphopeptides were analyzed using MALDI-TOF-TOF and ESI MS/MS (Table 2). Phosphopeptides from transferrin (S678, S409), unnamed protein containing MG1 domain

(S140), M2-type pyruvate kinase (S437), WD repeat-containing protein 59 isoform 3 (S375, Y437), ankyrin repeat domain 30B (S592), and coiled coil domain containing protein 73 (S770) were analyzed to show domain-specific phosphorylation sites, suggesting that protein phosphorylation could be influenced by surrounding amino acid sequences.

To establish the protein interactome map in the neuroretina and RPE, bioinformatics in AMD, bioinformatic STRING 10.0 software was used to integrate interaction data quantitatively from the STRING database from a large number of organisms and tissues. The STRING database currently covers 9,643,763 proteins from 2,031 organisms. AMD biomarker interactome was established using 94 proteins from the current phosphoproteome and published AMD proteome in the STRING query (Figure 3).

Oxidative stress mediated protein expression profile in the RPE

Next, to determine phosphorylation signaling in vitro, ARPE19 cells were treated with 200 μ M H₂O₂ or intense or constant light. Proteins were extracted and separated by two-dimensional electrophoresis and visualized by Coomassie blue staining. Proteins from PBS-treated RPE cells or RPE cells grown in normal light conditions were separated in parallel to serve as a control. Gel image overlay revealed that 17 protein spots were up-regulated and 7 proteins spots were down-regulated as a result of the H₂O₂ treatment (Table 3). Quantitative analysis of each spot under intense light (7,000–10,000 lux) or constant light (48 hours) based on normalized volume and intensity is listed in Table 3. Differentially expressed protein spots under various oxidative stress conditions were excised and digested by trypsin followed by protein identification using MALDI-TOF and MALDI-TOF-TOF mass spectrometry. Among these, retinoid metabolism related proteins, including RPE65, an all-trans-retinyl ester isomerohydrolase in the visual cycle, and retinol binding protein are of interest considering perturbed retinoid metabolism under oxidative stress in the RPE. Apoptotic (Annexin V) and redox reaction (GST) proteins were up-regulated and aging related (prohibitin) and respiratory (COX) proteins were down-regulated in RPE cells.

Potential biomarkers under oxidative stress were presented as an interaction network in Figure 4. Up- or down-regulated proteins under various stresses were added in STRING software query to establish a signaling network. The oxidative stress interactome showed potential connections between nuclear signaling (p53, BRCA1, ATM, SIRT1), oxygen-mediated apoptotic molecules (EPO, HIF, Bcl, BAD, TXN), and retrograde signaling (PHB, VDAC, EP300, RBP)-mediated reactions.

The analysis of AMD and oxidative stress interactomes revealed that 68–81% of protein signaling in AMD could be initiated from the oxidative stress biomarkers (Table 3; Figure 3 and 4). The protein interaction map supported the idea that protein phosphorylation could be involved in apoptosis the apoptotic or protective signaling in RPE cells. The AMD interactome (Figure 3) and oxidative stress biomarker interactome (Figure 4) suggest the following aspects of AMD: First, the AMD interactome indicated phosphocreatine shuttle alterations, suggesting changes of ATP-requiring reactions in AMD, maintenance of high ATP/ADP ratio, and rhodopsin phosphorylation. Further, AMD may require increased Na⁺/K⁺ ATPase, phosphatidylinositol/inositol triphosphate signaling, and cGMP metabolism. Creatine kinase may induce pumping out Na⁺ at night (dark) and blocking Na⁺

during the day (light). Creatine kinase catalyzes the major energy demanding reaction, whereas pyruvate kinase may regulate the pigmentation and phagocytosis [17,18]. In addition, ATP synthase could be involved in mitochondrial dysfunction.

Second, GFAP is a retinal stress indicator, controlling blood-retina barrier breakdown and initial changes of Müller cells and the RPE. We observed chaperone changes, including crystalline, heat shock proteins, and albumin as well as changes in endogenous antioxidants such as peroxiredoxin and thioredoxin, suggesting increased oxidative stress, peroxiredoxin, and thioredoxin in AMD.

Third, changes in phosphorylation pattern of retinoid binding proteins such as CRABP, CRALBP, RDH, RGR, RPE65, and IRBP. Altered expressions of retinoid binding protein changes may lead to perturbation in circadian clock, which is also suggested by cytoskeletal remodeling through actin, vimentin, and tubulin phosphorylation [8,19,20]. Cytoskeletal modifications can also be associated with changes in extracellular matrix regulated by MMPs and angiogenic reactions mediated by VEGF and EPO. Fourth, AMD donor tissue exhibited apoptotic changes in peripheral RPE. In particular, peripheral mitochondria were disrupted by depleted prohibitin as a nuclear-mitochondrial shuttle in the RPE. Fifth, AMD interactome map suggests altered lipid metabolism that includes cardiolipin as a mitochondrial indicator of apoptosis and cholesterol as a protein aggregator. Sixth, complement activation by C3, C9, CFB, CFH, and CFI, is observed in AMD via vitronectin, clustrin, and plasminogen interaction.

Correlation between AMD and Oxidative Stress Biomarkers

We determined whether there was a positive correlation between early apoptotic biomarkers in RPE cells under oxidative stress and the phosphoproteome changes in AMD using our *in vitro* and *in vivo* data including ARPE19 cells, animal models, and human donor eyes from AMD patients (Table 3). In Figure 5, the AMD interactome and oxidative stress were connected together using STRING 10.0 software to analyze the molecular mechanisms involved in AMD progression in terms of oxidative stress, inflammation switch, energy metabolism, and transcriptional regulation. The new AMD interactome with oxidative biomarkers demonstrated that several earlier unrelated to AMD proteins, including ubiquitin, peroxiredoxin, MAP kinase, BUB 1/3, vimentin and crystalline are involved within the AMD progression, suggesting that cytoskeletal protein phosphorylation, crystalline aggregation, and mitochondrial signaling may contribute to RPE apoptosis. Then to confirm oxidative stress biomarkers, specific cytoskeletal protein changes were determined *in vivo* using animal model (C3H female mice, 7 weeks old). Neurofilament, vimentin, and β -tubulin were upregulated under 24 hours constant light compared to 12 hours dark/12 hours light condition (data now shown). To address the issue of lipid composition in RPE cells, we determined lipid changes under oxidative stress (Table 4). Our data demonstrated that longer chain fatty acid at sn-2 in phosphatidylcholine increased from 16 carbons to 24/26 carbons as well as cholesteryl esters under oxidative stress. The saturation of fatty acid in phosphatidylcholine also increased.

Based on our proteomics data that identified altered signaling of apoptosis in the retina and RPE, the pathological pathway determined by the AMD interactome could yield suitable

targets for anti-apoptotic and anti-angiogenic therapy in Figure 6: (1) mitochondrial dysfunction in the peripheral RPE (prohibitin, ATP synthase); (2) oxidative stress including intense and constant light (peroxiredoxin, thioredoxin, glutathione S-transferase); (3) cytoskeletal remodeling by microtubule, actin filament, and intermediate filament (vimentin, actin, tubulin); (4) high concentration of nitric oxide (nitric oxide synthase); (5) hypoxia (HIF1, erythropoietin, VEGF); (6) disrupted circadian clock (melatonin); (7) apoptotic downstream (pJAK2, pSTAT3, Bclxl, caspases); (8) altered lipid concentrations (cardiolipin, cholesterol); (9) altered visual cycle (CRABP, CRALBP, RPE65); (10) altered energy metabolism (S/T vs Y kinases, carnitine, pyruvate, ATP synthase); (11) aggregation of heat shock proteins and crystallins; and (12) inflammation (CFH, C3, collagen, vitronectin).

DISCUSSION

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 AMD. Phosphoproteome changes under oxidative stress remain elusive in regard to the pathogenesis of AMD [21]. A detailed understanding of phosphorylation signaling in the RPE will answer how cytoskeletal reorganization and protein aggregation may control apoptosis and how we can intervene in the AMD apoptotic pathway.

Serial analysis of gene expression revealed 264 specific genes in rods, presenting a set of disease candidate genes and potential therapeutic targets. The set of rod-enriched genes suggested potential pathways in retinal biology, including electrophysiological responses of ion channels, phosphoinositide transfer, proteolysis, DNA damage repair, anti-oxidative enzymes, cytoskeletal components, and cadherins that bind nucleic acids [22]. Recently, cell-mediated immune response genes from RPE-choroid were identified in AMD [23,24]. Further, the top 20 genes as AMD biomarkers are predicted using a second donor cohort and a machine learning model. Transcriptome of human fetal RPE cells demonstrated that prolonged wound stimulus leads to passage-dependent altered expressions of the transcriptome, including TGF β activators. Global expression profiling determined RPE specific genes, including the visual cycle proteins, transporters, epithelial channels, and cell adhesion molecules [25].

Using reverse phase protein array, RPE exosomes showed signaling phosphoproteins under oxidative stress.[26] Stress-mediated exosome proteins are apoptosis and cell survival molecules, including Smac/Diablo, Akt, Src, ERK1/2, AMPK, and acetyl CoA carboxylate. The exosomes contain 38 phosphoproteins among 72 secreted proteins from RPE cells. This implies that the vesicle may have the potential to regulate signaling pathways in the retina and vitreous [26]. Mitochondrial complex I inhibition leads to increased autophagic markers and exocytotic activity, whereas decreased lysosomal activities in the RPE [27].

Our previous data suggested the potential cell death mechanisms in the retina and RPE under oxidative stress [8,9,12,13,16,28–30]. We demonstrated that oxidative stress may trigger induction of anti-apoptotic erythropoietin, JAK2, and BCL-xL, as well as pro-apoptotic caspases [28,29,31]. Oxidative stress also influenced mitochondrial-nuclear communication by shuttling mitochondrial prohibitin. In the current study, we observed that oxidative stress

regulated phosphorylations of cytoskeletal and anti-apoptotic proteins, including vimentin and crystallin, may serve as the early protective mechanism. The cytoskeletal network formed by filamentous polymers, including vimentin, actin, and tubulin, determines how retinal and RPE cells respond to their extracellular environmental stimuli that include intense light (>7,000 lux) or constant light (>one week).

Further, our proteomic approach to understand RPE cell death under stress conditions demonstrated 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, changed their expression, subcellular localization, phosphorylation, and lipid interaction; 4) relative lipid compositions, including phosphatidylcholine and cholesterol, were altered under oxidative stress; and 5) oxidative stress leads to cytoskeletal reorganization through site-specific vimentin phosphorylations that regulate intermediate filaments, resulting in nonfilamentous particles [32]. Oxidative stress may influence the expression of genetic risk factors of AMD, including complement factor H (CFH). Although the potential importance of 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 [33,34]. For example, abnormalities in vimentin phosphorylation have been linked to neurodegenerative diseases, including AMD and Alzheimer's disease, but the vimentin phosphorylation network mechanism in RPE cells under oxidative stress remains largely unknown [18,35,36].

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 (not 100% phosphorylated), and kinetics (short vs. long time frame). Understanding phosphorylation reaction in AMD is not clear, because protein phosphorylation is a very dynamic process, while the aging process is slow.

Pioneering proteomic profiling studies revealed the human RPE proteome, in addition to drusen, lipofuscin [37–39], native differentiated/cultured dedifferentiated RPE proteome [40,41], AMD proteome [3,4,42], and diabetic proteome [43]. Proteomic tools were used to study changes in the vitreous humor associated with diabetic retinopathy and retinal proteins in a glaucoma model [44]. 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 hurdles to get comprehensive details on the molecular mechanism of AMD.

In Vitro vs. In Vivo Phosphorylation

Previous data demonstrated that Hsp70 (c-Jun N-terminal kinase), crystallins (Akt), and the increased expression of VDAC might be involved in AMD progression [3,18,45,46]. Altered phosphorylations of mitochondrial heat shock protein mtHsp70, α A/ α B crystalline,

vimentin, and ATP synthase were observed in RPE cell death under oxidative stress [16,47]. Retinoid binding proteins, including CRABP, RPE65, and RLBP1, could be involved in the advanced stages of AMD [10,48–51]. We observed altered lipid compositions that include increased carbon number of fatty acids, double bond saturation, higher cholesterol, and phosphatidylcholine, whereas cardiolipin levels decreased. Changes in lipid concentrations seem to diminish the membrane fluidity and accelerate protein aggregation [52–54].

In vivo data demonstrated that PP2A and vimentin are modulated by constant light and are key elements involved in cytoskeletal signaling in rd1 mutation model [8]. The expression levels of vimentin and PP2A are significantly increased when C3HeB/FeJ mice (rd1 allele; 12 weeks; photoreceptors degenerated) are exposed under continuous light for 7 days compared to a condition of 12h light/dark cycling exposure. 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.

A positive correlation between the levels of PP2A and vimentin under light-induced stress suggests that cytoskeletal dynamics are regulated by vimentin phosphorylation. We postulate that light may induce post-translational modifications of vimentin and PP2Ac. We speculate that PP2Ac phosphorylation may control its subunit binding that determines its phosphatase activity and substrate specificity under stress conditions. Stabilized vimentin may act as an anti-apoptotic agent when cells are under stress.

The current interactome suggests that altered phosphoproteome interactions, including PP2A, pyruvate kinase, tyrosine kinase, and vimentin, exist in the retina and RPE in AMD. Phospho-Western blotting analysis revealed that phosphorylations of intermediate filament vimentin (Ser38, Ser55) and mitochondrial heat shock protein mt Hsp70 were modulated in the RPE in vitro [20]. Changes of vimentin phosphorylation are directed to reorganization of the intermediate filament network and altered function of RPE cells. The cytoskeletal network formed by filamentous proteins, including vimentin, determines how retinal and RPE cells respond to their extracellular environmental stimuli that include oxidative stress. Serine/threonine phosphatase PP2A-C α / β (Tyr) and tubulin α 1B/ β 2 were changed in the RPE, implying constant light-induced apoptosis signaling during AMD progression. Reducing the light-induced post-translational modification of vimentin and PP2A may assist to maintain the proper filament network in RPE cells.

Mechanistic Dissection of AMD

Recently, the locations and functions of crystallins in the retina and RPE have been described [47,55–57]. α A- and α B-crystallin are located in the ganglion cell layer and photoreceptor layer, whereas β -crystallin is detected in all nuclear layers of the retina [58]. The functional roles of crystallins as chaperones, anti-apoptotic proteins, or signal transducers in the retina have also been described [55,58–60]. Phosphorylated α B-crystallin directly interacts with Bax and caspase-3 to suppress their pro-apoptotic action, and thus

exerts a cytoprotective effect in the retina [60]. Light-induced up-regulation of α A-crystallin and increased phosphorylation of α A-crystallin in the aged retina have been reported. The specific function of each crystallin, particularly α/β -crystallin in the RPE, is still largely unknown, although levels of α B-crystallin are increased after heat shock and oxidative stress, and α B-crystallin immunoreactivity has been found in both rod outer segments and the RPE after light exposure [47,55,61].

Phosphoproteome alterations in apoptosis and angiogenesis in the retina and RPE may lead to the pathological pathway which would be suited as targets for anti-apoptotic and anti-angiogenic therapy: (1) mitochondrial dysfunction in the peripheral RPE; (2) oxidative stress including intense and constant light; (3) cytoskeletal remodeling; (4) high concentration of nitric oxide; (5) hypoxia; (6) disrupted circadian clock; (7) pJAK2, pSTAT3, Bclxl; (8) altered lipid concentrations; (9) altered visual cycle; (10) altered energy metabolism (S/T vs Y kinases, carnitine, pyruvate); and (11) heat shock proteins, crystallins. Based on our proteomics data, we tested the following anti-apoptotic or anti-angiogenic molecules: (1) prohibitin (anti-apoptotic mitochondrial-nuclear shuttle), (2) erythropoietin (anti-apoptotic protein via JAK2/STAT3 pathway), (3) melatonin (anti-apoptotic and anti-angiogenic molecule protecting cytoskeletal reorganization through PP2A/vimentin pathway), (4) okadaic acid, arginine, and SNAP to control nitric oxide concentration, (5) cardiolipin and cholesterol, (6) anthocyanin (anti-angiogenic via VEGFR2 pathway), (7) phospholipids, fatty acids, β -cyclodextrin to control lipids and cholesterol concentration [8,28].

The current study determined the changes of phosphoproteome in peripheral retina and the RPE from AMD eyes *in vivo*. Our data suggests that changes in phosphoprotein levels in response to oxidative stress are mechanistically important. We propose that the AMD mechanism is induced by the changes of protein expressions and aggregations by membrane remodeling and lipid oxidation. Phosphoproteins in AMD *in vivo* were compared to oxidative stress biomarker in the retina/RPE *in vitro*. Previous observations of vimentin derived from human choroidal neovascular membranes in AMD, as well as in drusen and melanolipofuscin, support the positive correlation between biomarkers in RPE cells under stress and the AMD proteome [62,63].

Our phosphoproteomic interactome provides new insights regarding how phosphoproteome is related to cytoskeletal changes. In addition, the AMD interactome map elucidates the regulatory mechanism of apoptotic cell death governed by phosphorylation. These outcomes are expected to have an important positive impact because inhibition of target phosphorylation might be assigned as therapeutic intervention to serve the physiological role for the balanced phosphorylated cytoskeleton and chaperone in RPE cells.

Phosphorylation-dependent signaling could be different in various tissues, including the retina and the RPE, and may reflect the microenvironment diversity among ocular cells. Changes in the global phosphoproteome may be indicative of an early signaling event under oxidative stress conditions in the RPE. Increased longer chain fatty acids in phosphatidylcholine, cholesteryl esters, and phosphorylated crystalline could contribute to AMD progression, considering that drusen contain lipids and oxidized proteins such as

crystallins. The aggregation mechanism by longer chain fatty acids and phosphorylated proteins may give insight into the molecular mechanism in AMD.

The current interactome map suggests that a positive correlation exists between early biomarkers of oxidative stress and phosphoproteins from AMD patients. The outcome of our work is the initial delineation of the underlying physiology of oxidative stress-mediated phosphorylation signaling in RPE apoptosis which can lead to AMD progression. In addition, the phosphoprotein interactome provides a stimulus for understanding oxidative stress-induced cytoskeletal changes and the mechanism of aggregate formation induced by protein phosphorylations. As a consequence, an effective therapeutic approach to treat AMD based on the modulation of phosphorylations are expected to result.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

AMD	age-related macular degeneration
DAPI	4',6-diamidino-2-phenylindole
DIGE	differential gel electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
EPO	erythropoietin
IP	immunoprecipitation; MMP
PP2A	protein phosphatase 2A
ROS	reactive oxygen species
RPE	retinal pigment epithelium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA

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Figure 1.

The senescence-associated molecular events during AMD progression were identified by comparing the phosphoproteome of macular (I) and peripheral (II) neurosensory retina, central RPE (III) and peripheral RPE (IV) from AMD eyes and age-matching control eyes. Phosphoproteins were enriched by charge-based spin column chromatography and resolved by two-dimensional gel electrophoresis. Trypsin-digested phosphorylated peptides were enriched using $\text{Ga}^{3+}/\text{TiO}_2$ immobilized metal ion chromatography. Eluted phosphopeptides

were analyzed by mass spectrometry. Statistical analysis and comparison show that altered phosphorylation signaling exists in AMD.

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Figure 2.

A. Phosphoproteins from peripheral retina in AMD separated by 2D gel electrophoresis. Each spot represents Coomassie blue stained phosphoproteins (200 μ g) from AMD eyes or age-matching control from the peripheral retinal region, which indicates the phosphorylation changes in AMD. Protein spots in the box with number represent identified proteins with altered phosphorylations. Y axis represents linear range of pI 4–6.5 and 7.5 to 9.5, and Y axis shows molecular weight (10–75 kDa). **B.** Phosphoproteins from retinal pigment epithelium (RPE) of AMD eyes separated by 2D gel electrophoresis. Each spot represents

Coomassie blue stained phosphoproteins (200 µg) from the peripheral RPE region, which indicates the changes of phosphorylation signaling in AMD. Protein spots in the box represent identified protein by mass spectrometry analysis with altered phosphorylation. Y axis represents linear range of pI 4–6.5 and 7.5 to 9.5, and Y axis shows molecular weight (10–75 kDa).

12 Transferrin

13 Pyruvate kinase

14 Enolase

15 Unnamed protein (α2 macroglobulin MG1 domain)

16 M2 Pyruvate kinase

17 PAK S/T kinase

17 PP2A 19, 24 Unnamed protein

21 GFAP

22 Unnamed protein (Tyrosine protein kinase FES/FPS)

23 Dynamin-like protein

25 Creatine kinase

26 Creatine kinase

29 Vimentin

30 Pyruvate kinase

Iron homeostasis

Energy regeneration: Glycolytic flux

Intermediate filament: Constant light

High energy fluctuation

Energy shuttle

31, 34 Unnamed protein

32, 35 Unnamed protein (NADP ubiquinon oxidoreductase)

37 Unnamed protein (Inositol 1,4,5triphosphate receptor)

39 Unnamed protein

41, 43 Unnamed protein (Calponin) Ankyrin repeat domain 30B

42, 45 Guanylate cyclase Unnamed protein (Sorting nexin 13)

44 Coiled-coil domain protein 73

Fast energy transition

Oxidative damage

Blood flow

Physiological hypoxia

cGMP

Apical polarity

Figure 3. AMD Biomarker Interactome

Potential biomarkers of AMD were presented as an interaction network. Proteins found in AMD eyes were added in STRING software query and established signaling pathways. Protein-protein interaction network was established by bioinformatics tools that include Osprey, VISANT, Navigator, STRING, iHOP, and Expasy protein database. STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations. Interactions are derived from eight sources that include genomic context, high-throughput interaction experiments, conserved co-expression, and published knowledge (Pubmed, MiPS). STRING quantitatively integrates interaction data from these sources for a large number of organisms, and transfers

information between these organisms where applicable. The database currently covers 9,643,763 proteins from 2,031 organisms. AMD biomarker interactome suggests that 68–81% of protein signaling in AMD could be initiated under oxidative stress. Interactions of complement factors (C3, C9, CFH, vitronectin), retinoid binding proteins (RPE65, RGR, RLBP), apoptotic molecules (Bcl2, BAD, BAX, BAK), energy metabolism related mitochondrial proteins (ATPase), transcription (p53), protease (MMP), cytoskeletal polymers (lamin, albumin), are noticed.

Protein-Protein Interaction

- Neighborhood
- Gene Fusion
- Cooccurrence
- Coexpression
- Experiments
- Databases
- Textmining
- Homology

Figure 4. Oxidative Stress Interactome

Potential biomarkers of oxidative stress in the retina and RPE were presented as an interaction network. Up- or down-regulated proteins under oxidative stress were added in STRING software query and established signaling pathways. Interactions in nuclear signaling (p53, BRCA1, ATM, SIRT1), oxygen-mediated apoptotic molecules (EPO, HIF, Bcl, BAD, TXN), retrograde signaling (PHB, VDAC, EP300, RBP), are observed.

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Figure 6.

Mechanistic dissection of AMD network. Based on our proteomics data, the potential AMD mechanism is proposed: (1) mitochondrial dysfunction in the peripheral RPE (prohibitin, ATP synthase), (2) oxidative stress including intense and constant light (peroxiredoxin, thioredoxin, glutathione S-transferase), (3) cytoskeletal remodeling by microtubule, actin filament and intermediate filament (vimentin, actin, tubulin), (4) high concentration of nitric oxide (nitric oxide synthase), (5) hypoxia (HIF1, erythropoietin, VEGF), (6) disrupted circadian clock (melatonin), (7) apoptotic downstream (pJAK2, pSTAT3, Bclxl, caspases)

(8) altered lipid concentrations (cardiolipin, cholesterol), (9) altered visual cycle (CRABP, CRALBP, RPE65), (10) altered energy metabolism (S/T vs Y kinases, carnitine, pyruvate, ATP synthase), (11) aggregation of heat shock proteins and crystallins, (12) inflammation (CFH, C3, collagen, vitronectin).

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Table 1**Enriched Phosphoproteins from AMD eyes**

Phosphoproteins from the retina and the RPE of AMD and age-matching control eyes were separated by 2D gel electrophoresis. Up- or down-regulated protein in AMD or age-matching control were digested using trypsin and analyzed by MALDI-TOF-TOF and ESI tandem mass spectrometry. Spot #, identified proteins, MOWSE score, % coverage, theoretical molecular weight, isoelectric point, number of identified peptides are shown. MOWSE score is expressed as $-10\log P$ as a probability value to compute the composite probability P.

Spot #	Protein identified	MOWSE score	% coverage	Mass	pI	Peptide identified
12	transferrin	1.94e+12	28.7	76961	7.0	19
13	pyruvate kinase isozymes M1/M2 isoform e	195852	19.8	56274	8.7	8
14	2-phosphopyruvate-hydratase alpha-enolase	2.10e+10	28.6	47109	7.0	10
15	2-phosphopyruvate-hydratase alpha-enolase	7.22e+7	28.6	47109	7.0	10
16	M2-type pyruvate kinase	430810	15.8	57914	8.0	7
19	Unnamed (primary human renal epithelial cells)	1488	9.2	58545	8.7	4
21	Glial fibrillary acidic like protein	1.80e+10	38.8	47337	5.3	14
22	tyrosine-protein kinase Fes/Fps	448928	17.0	93779	6.3	11
22	receptor tyrosine kinase	412210	12.5	99657	6.9	7
23	Unnamed (Hsp70 protein)	219797	14.2	68116	5.3	7
23	dynammin-like protein Dymple isoform	5.08e+6	13.6	78062	6.3	7
25	creatine kinase, brain, isoform CRA_a	2.33e+6	27.3	42528	5.3	7
26	GAPDH (GTPase activating protein)	5.67e+6	12.0	96010	7.3	8
26	WD repeat-containing protein 59 isoform 3	2.38e+6	13.3	109801	8.0	7
27	Unnamed (intermediate filament head)	171525	18.0	47488	5.0	7
35	unnamed protein (NADH					
	Ubiquinone oxidoreductase, 20 Kd subunit)	8961	34.5	22203	9.9	4
39	switch-associated protein 70	110691	8.0	68998	5.7	5
40	ankyrin repeat domain 30B	79.6	5.1	91935	5.9	5
43	Coiled coil domain containing protein 73	14699	12.8	92721	5.2	5
44	WD repeat-containing protein 64	92874	8.2	123627	6.7	5

Table 2
Enriched Phosphopeptides in AMD

Trypsin digested phosphopeptides were enriched using Ga³⁺/TiO₂ immobilized metal ion chromatography. Eluted phosphopeptides were analyzed using MALDI-TOF-TOF and ESI MS/MS. Some protein spots contained more than one protein such as spot 15.

Spot	Protein Identified	Potential modification	Peptide	Position
12	transferrin	Phosphorylation	CSTSSLLEACTFR	S678
			IMNGEADAMSLDGGFVYIAGK	S409
15	unnamed protein (MG1 domain)	Phosphorylation	IPITYNNGILFIHTDKPVYTPDQSVK	S140
16	M2-type pyruvate kinase	Phosphorylation	SAHQVAR	S437
26	WD repeat-containing protein 59 isoform 3	Phosphorylation	EDPPSSLLEEK	S375
			MLVTFPAQYPNNAAPSFQFINPTTITSVK	Y437
43	ankyrin repeat domain 30B	Phosphorylation	LEESPVK	S592
44	coiled coil domain containing protein 73	Phosphorylation	AEPLISTVQQSHSQTVK	S770

Table 3

Comparison of Proteome *In Vivo* vs. *In Vitro*

Proteins found in AMD compared to altered proteins under oxidative stress. Molecular components, potential biomarkers and reactions of AMD (I-X) are presented under the table. Color codes represent whether the identical protein (pink) or discrete function (green) between AMD biomarkers *in vivo* vs. oxidative stress biomarkers *in vitro*. Protein list was obtained from the following references: [1] Nordgaard et al., IOVS, 2006, 47, 815 [2] Crabb et al., PNAS, 2002, 12, 14682 [3] Alcazar et al., MCP, 2009, 8, 2201 [4] Arnouk et al., JOP, 2011, 74, 254 [5] Lee et al., IJBM, 2011, 48, 194 [6] Ethen et al., IOVS, 2006, 47, 2280 [7] An et al., JPR, 2006, 5, 2599 [8] Warburton et al., MV, 2007, 13, 318 [9] Chung et al., JNR 2009, 87, 2365 [10] Zhang et al., IJBM, 2010, 47, 255

AMD-related Proteome		Retina/RPE proteome under OS			
Protein	Model	Reference (category)	Proteins	Model	Reference (category)
2-phosphopyruvate-hydratase alpha-enolase	AMD RPE	current study	Actin	RPE light	5(V)
2-phosphopyruvate-hydratase alpha-enolase	AMD RPE	current study	Actin	Retina proteome down melatonin	9(V)
Ankyrin repeat domain 30B	AMD RPE	current study	Alpha-crystallin A chain	Retina proteome down melatonin	9(III)
Annexin 5	AMD drusen	2(VI)	Alpha-crystallin A chain	Retina proteome down melatonin	9(III)
Annexin 5	RPE blebs	3(VI)	Alpha-crystallin A chain	Retina proteome down constant light	9(III)
Annexin 5	RPE secretome	6(VI)	Alpha-crystallin B chain	Retina proteome down melatonin	9(III)
Annexin 5	Melanolipofuscin	8(VI)	Alpha-crystallin B chain	Retina proteome down constant light	9(III)
ATP synthase	Melanolipofuscin	8(I)	Annexin 5	RPE proteome OS	4(VI)
ATP synthase b	AMD RPE	1(I)	Aspartate aminotransferase	Retina proteome down constant light	9(I)
Caspase 5	RPE secretome	7(VI)	ATP synthase	RPE light	5(I)
CFB	RPE secretome	7(VIII)	BCLx1	Intense light	10(VI)
CFB	AMD secretome	7(VIII)	Beta-crystallin A1	Retina proteome down melatonin	9(III)
CFH	RPE secretome	7(VIII)	Beta-crystallin A1	Retina proteome down constant light	9(III)
CFH	AMD secretome	7(VIII)	Beta-crystallin A2	Retina proteome down melatonin	9(III)
Clusterin	AMD drusen	2(VI)	Beta-crystallin B1	Retina proteome down constant light	9(III)

AMD-related Proteome		Retina/RPE proteome under OS			
Protein	Model	Reference (category)	Proteins	Model	Reference (category)
Coiled coil domain containing protein 73	AMD RPE	current study	Beta-crystallin B3	Retina proteome down constant light	9(III)
	AMD secretome	7(v)	Beta-crystallin S	Retina proteome down constant light	9(III)
	RPE secretome	7(vIII)	Beta-soluble NSF attachment protein	Retina proteome down melatonin	9
	AMD secretome	7(vIII)	BUB3	RPE proteome OS	4
Complement C	AMD drusen	2(vIII)	Caspase 3	Intense light	10(vI)
Complement C	AMD retina	6(IV)	cFOS	Intense light	10
Complement component 9	AMD RPE	1(IV)	CRALBP	RPE light	5
	AMD RPE	1(IV)	Creatine kinase	RPE light	5(III)
	AMD RPE	current study	Crystallin aA	RPE light	5(III)
	AMD RPE	1(III)	Crystallin aB	RPE light	5(III)
creatine kinase, brain, isoform CRA_a	AMD retina	6(III)	Crystallin bB	RPE light	5(III)
	AMD drusen	2(III)	Crystallin g	RPE light	5(III)
	AMD retina	6(III)	Dihydropyrimidinase-related protein 2	Retina proteome up constant light	9
	Melanolipofuscin	8(III)	Dihydropyrimidinase-related protein 2	Retina proteome down melatonin	9
Crystallin aB	AMD drusen	2(III)	EF2	RPE proteome OS	4
Crystallin bA3	AMD secretome	7(vI)	Erythropoietin	Circadian	10(v)
Clusterin	RPE blebs	3(v)	Erythropoietin receptor	Circadian	10(v)
	RPE blebs	3(v)	G binding protein b1	RPE light	5(1)
Cytoskeleton-associated protein 4	AMD RPE	current study	Gamma crystallin B	Retina proteome down constant light	9(III)
	RPE blebs	3	Gamma-crystallin F	Retina proteome down constant light	9(III)
dynamitin-like protein Dymple isoform	AMD RPE	current study	GST p	RPE proteome OS	4(1)
EF 1	RPE blebs	1	Guanine binding protein	RPE proteome OS	4(1)
Elf4H	AMD RPE	8(1)	Guanine nucleotide-binding protein G(I)G(S)/G(T) subunit b-3	Retina proteome down melatonin	9(1)
G binding protein	Melanolipofuscin	7			
Galectin 3 BP	AMD secretome				

AMD-related Proteome		Retina/RPE proteome under OS			Reference (category)	
Protein	Model	Proteins	Model	Reference (category)		
GAP111 (GTPase activating protein)	AMD RPE	Guanine nucleotide-binding protein G (o) subunit alpha	Retina proteome up constant light	9(I)		
Glial fibrillary acidic like protein	AMD retina	Guanine nucleotide-binding protein G(o) alpha	Retina proteome down melatonin	9(I)		
GST	RPE blebs	HIF1A	Hypoxia retina/RPE	10		
GST p	AMD RPE	HO-1	Intense light RPE	10		
GTPase Rab 14	RPE blebs	HSP 70	RPE light	5(III)		
Histone H2A2	AMD drusen	HSP 90	RPE light	5(III)		
HSC 70	AMD RPE	HSP 90 a	RPE proteome OS	4(III)		
HSP 47	RPE secretome	HSP b1	RPE proteome OS	4(III)		
HSP 60	AMD RPE	HSP b1	RPE light	5(III)		
HSP 60	AMD retina	Inorganic pyrophosphatase	Retina proteome down melatonin	9(I)		
HSP 60	Melanolipofuscin	IRBP	RPE light	5(IV)		
HSP 70	AMD RPE	Lamin A/C	RPE proteome OS	4(V)		
HSP 70	Melanolipofuscin	Mitochondrial inner membrane protein	Retina proteome down melatonin	9(VI)		
HSP 70 9B	RPE blebs	Nucleoside diphosphate kinase A	Retina proteome up constant light	9(I)		
HSP70	AMD retina	Peroxiredoxin 1, 2	RPE proteome OS	4(III)		
Keratin 7, 18	RPE blebs	Peroxiredoxin-2	Retina proteome up constant light	9(III)		
Lamin A/C	RPE blebs	Peroxiredoxin-6	Retina proteome up constant light	9(III)		
Laminin	RPE secretome	Phosphoglycerate mutase 1	Retina proteome up constant light	9(I)		
M2-type pyruvate kinase	AMD RPE	Phosphomevalonate kinase	RPE proteome OS	4(I)		
MMP-14	RPE blebs	pJAK2	Hypoxia	10(X)		
MMP2	RPE secretome	pJAK2	Hyperoxia	10(X)		
MMP2	AMD secretome	pJAK2	Intense light	10(X)		
Mt HSP 75	AMD RPE	Plasminogen	RPE light	5(X)		

AMD-related Proteome		Retina/RPE proteome under OS			
Protein	Model	Reference (category)	Proteins	Model	Reference (category)
Peroxiredoxin 5	RPE blebs	3(III)	Plasminogen activator- inhibitor 1	RPE proteome OS	4(III)
Prasminogen activator inhibitor 1	RPE secretome	7	Prohibitin	RPE proteome OS	4(VI)
Prohibitin	Melanolipofuscin	8(VI)	Protein kinase 4	RPE light	5
Pyruvate kinase	AMD RPE	1(I)	Pyruvate kinase	RPE proteome OS	4(I)
pyruvate kinase isozymes M1/M2 isoform e	AMD retina	current study	Pyruvate kinase	RPE light	5(I)
RBP 3	Melanolipofuscin	8(IV)	Rab GDP dissociation inhibitor alpha	Retina proteome up constant light	9(I)
RDH 11	Melanolipofuscin	8(IV)	Rab GDP dissociation inhibitor alpha	Retina proteome down melatonin	9(I)
RDH 5	Melanolipofuscin	8(IV)	Retinaldehyde-binding protein 1	Retina proteome up constant light	9
RGR	Melanolipofuscin	8(IV)	Retinaldehyde-binding protein 1	Retina proteome down melatonin	9
RPE65	Melanolipofuscin	8(IV)	Retinol binding protein	RPE proteome OS	4
Serum albumin	AMD drusen	2(III)	RPE65	RPE proteome OS	4(IV)
switch-associated protein 70	AMD RPE	current study	RPE65	RPE light	5(IV)
Thioredoxin reductase 1	RPE blebs	3(III)	Ser/Thr-protein phosphatase 2A 65 kDa regulatory subunit A α	Retina proteome up constant light	9(I)
TIMP3	AMD drusen	2(X)	Ser/Thr-protein phosphatase 2A 65 kDa regulatory subunit A α	Retina proteome down melatonin	9(I)
transferrin	AMD retina	current study	Serum albumin precursor	Retina proteome down melatonin	9(III)
Tubulin	RPE blebs	3(V)	Thioredoxin	Intense light RPE	10(III)
Tubulin a	AMD retina	6(V)	Thioredoxin-dependent- peroxide reductase 3	RPE proteome OS	4(III)
Tubulin a	Melanolipofuscin	8(V)	Triosephosphate isomerase	Retina proteome down constant light	9
Tubulin b	Melanolipofuscin	8(V)	Tubulin a1B	RPE light	5(V)
Tyrosine-protein kinase Fes/Fps	AMD retina	current study	Tubulin b2	RPE light	5(V)
Ubiquinone oxidoreductase, 20 Kd subunit)	AMD RPE	current study	Tubulin beta-5 chain	Retina proteome up constant light	9(V)
Unnamed (Hsp70 protein)	AMD RPE	current study	VDAC2	RPE proteome OS	4(VI)

AMD-related Proteome		Retina/RPE proteome under OS			
Protein	Model	Reference (category)	Proteins	Model	Reference (category)
Unnamed (intermediate filament head)	AMD RPE	current study	Vesicle-fusing ATPase	Retina proteome up constant light	$\mathcal{Q}_{(I)}$
Unnamed (primary human renal epithelial cells)	AMD RPE	current study	vimentin	Retina proteome up constant light	$\mathcal{Q}_{(V)}$
Unnamed protein (NADH Ubiquinone oxidoreductase)	AMD RPE	current study	vimentin	Retina proteome down melatonin	$\mathcal{Q}_{(V)}$
VDAC 1	AMD RPE	$I_{(VI)}$			
VDAC 1, 2, 3	Melanolipofuscin	$\delta_{(VI)}$			
VDAC1	AMD retina	$\alpha_{(VI)}$			
VDAC3	RPE blebs	$\beta_{(VI)}$			
Vimentin	Melanolipofuscin	$\delta_{(V)}$			
Vimentin	AMD drusen	$\hat{2}_{(V)}$			
Vitronectin	AMD drusen	$\hat{2}_{(X)}$			
WD repeat-containing protein 59 isoform 3	AMD retina	current study			
WD repeat-containing protein 64	AMD retina	current study			

References

1 Nordgaard et al., IOVS, 2006, 47, 815
2 Crabb et al., PNAS, 2002, 12, 14682
3 Alcazar et al., MCP, 2009, 8, 2201
4 Arnouk et al., JOP, 2011, 74, 254
5 Lee et al., IJBM, 2011, 48, 194
6 Ethen et al., IOVS, 2006, 47, 2280
7 An et al., JPR, 2006, 5, 2599
8 Warburton et al., MV, 2007, 13, 318
9 Chung et al., JNR 2009, 87, 2365
10 Zhang et al., IJBM, 2010, 47, 255

Category

I. Phosphocreatine Shuttle Changes in AMD

- 1 Changes of ATP-requiring reactions in AMD
- 2 Maintenance of high ATP/ADP ratio
- 3 Rhodopsin phosphorylation
- 4 Na^+/K^+ ATPase
- 5 Phosphatidylinositol/inositol triphosphate signaling
- 6 cGMP metabolism
- 7 Pumping out Na^+ (night), blocking Na^+ (day)
- 8 Creatine kinase (major energy source)vs. pyruvate kinase (pigmentation, phagocytosis) vs. ATP synthase (mitochondrial dysfunction)

II. GFAP as a Retinal Stress Indicator

- 1 Blood-retinal barrier breakdown
- 2 Initial changes of Müller cell and RPE

III. Chaperone Changes

- 1 Crystalline, heat shock proteins
- 2 Albumin
- 3 Oxidative stress, peroxiredoxin, thioredoxin

IV. Altered Retinoid Metabolism

- 1 CRABP, CRALBP
- 2 RDH, RGR, RPE65, IRBP

V. Circadian Clock

- 1 Vimentin, PP2A
- 2 Cytoskeletal remodeling, tubulin, phosphorylation changes
- 3 Extracellular matrix, MMP, angiogenesis, EPO

VI. Apoptotic Peripheral RPE

- 1 Prohibitin as a nuclear/mitochondrial shuttle in the retina/RPE
- 2 Lipid binding switch
- 3 Mitochondrial dysfunction

VII. Lipid Metabolism

- 1 Cardiolipin as a mitochondrial indicator of apoptosis
- 2 Cholesterol as an aggregator

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VIII. Complement Activation	Fatty acids balance	
	3	
IX. Unknown Proteins	1	Unnamed proteins
	2	Homologous proteins
X. RPE Aging	1	Protein fragmentation, aggregation
	2	Retina overproliferation vs. RPE apoptosis

Table 4

Lipid Composition Changes in the RPE under Oxidative Stress

Lipids changes under oxidative stress (H_2O_2 200 μM , 6 hrs) in ARPE-19 cells. M/Z presents mass over charge and relative intensity shows relative concentration of ion peak compared to the most abundant base peak.

m/z	Rel. Int.	Control Lipid	Fatty acid	m/z	Rel. Int.	Oxidative stress Lipid	Fatty acid
1543.8	22.09	phosphoethanolamine	18:1c9/20:1c11	637.3	33.74	phosphatidylglycerol	O-14:0/O-14:0
1465.9	18.2	glycerol, nonitol	32:08:00	734.6	30.29	phosphatidylcholine	12:0/20:0
1542.9	17.49	Phosphoethanolamine Salt	16:0/16:0	810.5	29.58	phosphatidylcholine	18:0/20:4c5,8,11,14
1549.9	14.87	phosphatidylcholine	18:0/18:1c9,12	1518.8	25.55	phosphatidylcholine	32:0/2-phy./2-phy.-Tet
298.8	14.37	sphingosine		808.6	25.31	phosphatidic acid	18:0/26:2y10,12
1508	14.16	phosphatidylcholine	14:0/20:4c5,8,11,14	762.7	21.25	phosphatidylcholine	10:0/24:0
734.6	13.74	phosphatidylcholine	12:0/20:0	902.6	20.71	phosphatidylcholine	18:0/26:0
808.6	13.54	phosphatidic acid	18:0/26:2y10,12	876.6	20.62	phosphatidylcholine	16:0/26:0
783.6	10.91	phosphatidylcholine	18:2c5,9/18:2c5,9	783.6	20.53	phosphatidylcholine	18:2c5,9/18:2c5,9
637.4	10.06	phosphatidylglycerol	O-14:0/O-14:0	836.6	19.2	phosphatidylethanolamine	22:6c4,7,10,13,16,19
639.6	1.7	cholesteryl ester	methyl palmitate	850.6	18.34	phosphatidylcholine	16:0/10:1pyr.10
651.5	1.55	cholesteryl ester	oleate	787.6	18.31	phosphatidylcholine	18:0/18:2c9,12
386.2	0.93	cholesterol		792.5	18.1	phosphatidylcholine	12:0/24:0
				651.6	5.02	cholesteryl ester	oleate
				639.5	3.93	cholesteryl ester	methyl palmitate
				386.1	1.83	cholesterol	