Published in final edited form as:
J Proteome Res. 2010 February 5; 9(2): 1173. doi:10.1021/pr900819k.

Proteomic Changes in the Photoreceptor Outer Segment Upon Intense Light Exposure

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Abstract

Acute light-induced photoreceptor degeneration has been studied in experimental animals as a model for photoreceptor cell loss in human retinal degenerative diseases. Light absorption by rhodopsin in rod photoreceptor outer segments (OS) induces oxidative stress and initiates apoptotic cell death. However, the molecular events that induce oxidative stress and initiate the apoptotic cascade remain poorly understood. To better understand the molecular mechanisms of light-induced photoreceptor cell death, we studied the proteomic changes in OS upon intense light exposure by using a proteolytic ^{18}O labeling method. Of 171 proteins identified, the relative abundance of 98 proteins in light-exposed and unexposed OS was determined. The quantities of 11 proteins were found to differ by more than 2-fold between light-exposed OS and those remaining in darkness. Among the 11 proteins, 8 were phototransduction proteins and 7 of these were altered such that the efficiency of phototransduction would be reduced or quenched during light exposure. In contrast, the amount of OS rhodopsin kinase was reduced by 2-fold after light exposure, suggesting attenuation in the mechanism of quenching phototransduction. Liquid chromatography multiple reaction monitoring (LC-MRM) was performed to confirm this reduction in the quantity of rhodopsin kinase. As revealed by immunofluorescence microscopy, this reduction of rhodopsin kinase is not a result of protein translocation from the outer to the inner segment. Collectively, our findings suggest that the absolute quantity of rhodopsin kinase in rod photoreceptors is reduced upon light stimulation and that this reduction may be a contributing factor to light-induced photoreceptor cell death. This report provides new insights into the proteomic changes in the OS upon intense light exposure and creates a foundation for understanding the mechanisms of light-induced photoreceptor cell death.

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Keywords

photoreceptor; light damage; ^{18}O labeling; mass spectrometry; rhodopsin kinase; phototransduction

Introduction

Acute light-induced photoreceptor cell degeneration has been studied in experimental animals for over 40 years as a model for retinal cell loss arising from human retinal degenerative diseases.[1] A number of experiments have suggested that light-induced photoreceptor degeneration begins with absorption of light by rhodopsin in the photoreceptor outer segments (OS).[2-5] While this rhodopsin "bleaching" activates transducin as a part of normal vision, excessive activation may trigger photoreceptor degeneration.6 In addition to extensive transducin activation, photo-oxidative stress is another potential mechanism to induce photoreceptor degeneration. Administration of antioxidants reduces the extent of rhodopsin mediated photo-oxidative stress and prevents photoreceptor cell death.[7-11] Although the light absorption step in photo-oxidative stress is reasonably well characterized, the molecular events that mediate or regulate the subsequent apoptotic cascade remain poorly understood.

Photoreceptor cells have evolved to maximally absorb visible light, which occurs in the OS, a photoreceptor cell sub-compartment. OS is filled with stacks of photosensitive membranes (disks) that contain the visual pigment rhodopsin and much of the phototransduction machinery embedded within a highly unsaturated phospholipid bilayer. In the dark adapted state, photoreceptor opsin apoprotein is bound with 11-*cis*-retinal to form rhodopsin. Rhodopsin comprises more than 80% of total OS membrane protein,[12] and forms paracrystalline arrays in disk membranes of rodent photoreceptors.[13] Rhodopsin concentration can reach 4.6 mM in disk membranes,[14] which may contribute to the retina's susceptibility to light damage. The availability of proteins involved in quenching the phototransduction cascade,[15] such as arrestin[16, 17] and rhodopsin kinase,[17] has been shown to be important for protection from light-induced damage. Deactivation of rhodopsin, mediated by rhodopsin kinase and arrestin, is one of the rate limiting steps in the phototransduction cascade.[18] To understand how the photo-susceptibility of photoreceptors is modulated during and after induction of light damage, it is essential to quantitatively characterize these proteins involved in quenching phototransduction.

Light exposure causes certain proteins to translocate between the OS and other photoreceptor compartments.[19] Given the dynamic subcellular re-localization of these proteins, examination of retinal mRNA levels provides only limited information about their quantities in OS. While Western blot analysis and immunohistochemistry can be used to quantify OS proteins, these antibody-based techniques are low-throughput and require foreknowledge of the proteins being analyzed. Among the methods currently available, the proteomic approach is the only high-throughput method that does not require foreknowledge of proteins expressed in biological systems. However, proteomic analysis of OS proteins is challenging because many proteins involved in phototransduction are either integral membrane proteins or proteins modified by lipids. Since those membrane associated proteins are hard to solubilize, 2D-gel electrophoresis-based methods are not compatible with OS samples. Indeed, only a few proteomic studies examining this cell compartment have been reported so far,[20-22] with none taking a quantitative approach.

To gain insight into the mechanisms that may contribute to light-induced photoreceptor degeneration, we compared OS proteins from light-exposed and unexposed rats with a proteolytic ^{18}O labeling method.[23] Of 98 proteins that were quantitatively analyzed, 11 proteins showed significant changes upon light exposure. Among the significantly changed proteins,

rhodopsin kinase was further analyzed by liquid chromatography multiple reaction monitoring (LC-MRM) and immunofluorescence microscopy.

Materials and Methods

Materials

Oxygen-18 enriched water was obtained from Cambridge Isotope Laboratories (Andover, MA) or from Isotec (Miamisburg, OH). Sequencing grade modified porcine trypsin was purchased from Promega (Madison, WI). All other chemicals and materials were either reagent grade or were of the highest quality that was commercially available. All carbon-13 (^{13}C), nitrogen 15 (^{15}N) labeled peptides were synthesized by Sigma Genosys (The Woodlands, TX) or AnaSpec (San Jose, CA).

Light exposure and harvesting of rat retinal tissue

Male Sprague-Dawley weanling rats (Harlan Inc., Indianapolis, IN) were reared in a dim (20-40 lux) cyclic light environment (lights were on at 8 AM and off at 8 PM) for at least 40 days. The rats were fed standard rat chow (Teklad, Madison, WI) and given water *ad libitum*. After the 40 day cyclic light rearing period, randomly selected animals were assigned to control (4 rats × 4 replicates) or experimental (4 rats × 4 replicates) groups and were dark adapted for 16 h. After dark adaptation, the experimental group was exposed to bright green light (wavelength range 490-580 nm, light intensity 1200 Lux) for 8 h starting at 1 AM in animal treatment chambers made from cylindrical green plexiglas (Cat # 2092, Dayton Plastic, Dayton, Ohio).[24] This duration and intensity of light has been shown to damage photoreceptors such that it results in approximately 50% photoreceptor loss when measured 2 weeks later.25 The control group remained in darkness for the same 8 h. The rats were sacrificed in a chamber with a CO_2-saturated atmosphere under dim red illumination immediately after the 8 h light or dark period. Retinas were excised under dim red illumination within two minutes of death and rinsed with phosphate buffered saline (PBS). It should be noted that because the process of light-induced photoreceptor cell death occurs over several days to 2 weeks, photoreceptor cell loss immediately after light exposure is negligible.[9]

Preparation of photoreceptor outer segments

Retinas from four rats were combined and used to prepare and isolate photoreceptor OS by sucrose density ultracentrifugation.[24] Following isolation, the purified OS were stored at −80° C until use. All solvents used for rat OS preparations contained protease inhibitors (1 mM EDTA, 0.2 mM PMSF, 0.7 μg/μl leupeptin, and 0.5 μg/μl pepstatin A) and 100 μM diethylenetriamine pentaacetic acid (DTPA) to inhibit protein degradation and oxidation, respectively. Only band I fractions, which represent the purest OS preparation,[26] were used in this study. To prevent possible light-induced protein migration *in vitro*, retinal dissections and OS preparations were done under dim red light.

Proteolytic ^{18}O labeling

The isolated OS material was stored in a 37% sucrose solution (300 μL), then mixed with an equal volume of PBS containing 20% sucrose, followed by centrifugation at 10,000 g for 5 min using a refrigerated tabletop centrifuge at 4°C. The precipitated OS were dissolved in 30 μL of 2% sodium dodecyl sulfate (SDS) in 50 mM ammonium bicarbonate, and the extracted proteins were reduced by 10 mM DTT and *S*-alkylated by 25 mM iodoacetamide.[27] The reduced and *S*-alkylated proteins were then precipitated by mixing with a 6-fold excess volume of ice-cold acetone and by incubation for at least 2 h at −20°C. The precipitated protein was then centrifuged at ~1,000 g for 15 s in a tabletop centrifuge and washed twice with ice-cold acetone to remove excess SDS. The protein pellet was redissolved in 0.1% acid cleavable

surfactant, Rapigest[28] (w/v) (Waters Corporation, Milford, MA), in 50 mM ammonium bicarbonate. The amount of protein dissolved in 0.1% Rapigest solution was determined by using the DC Protein assay kit (Bio-Rad, Hercules, CA). Each OS sample contained approximately 25 μg of protein.

The protein concentrations in control and light treated samples were equalized with 0.1% Rapigest solution in 50 mM ammonium bicarbonate before digestion. The light exposed and unexposed OS sample proteins were digested separately in $H_2{}^{16}O$ by trypsin (1:100 substrate to protein ratio, w/w) at 25°C for 18 h. Following digestion, Rapigest was cleaved by adding formic acid (final concentration 0.1% (v/v)) and incubating at 40°C for 2 h. The resulting turbid solution containing tryptic peptides was loaded onto a Vydac C18 column (The Nest Group, Inc., Southborough, MA) and desalted according to the manufacturer's directions. The peptides were eluted from the column using 60% (v/v) acetonitrile containing 0.1% formic acid and dried in a Speed-vac concentrator. The dried peptides from light-exposed and unexposed OS were dissolved in 100 mM citrate buffer pH 6 (50 μL) made with $H_2{}^{16}O$ and $H_2{}^{18}O$, respectively. The peptides were then incubated with trypsin (1:25 substrate to protein ratio, w/w) at 25°C for 18 h to incorporate ^{16}O and ^{18}O, respectively, into the carboxyl termini of the peptides. This acidic pH condition has been demonstrated to provide higher ^{18}O labeling efficiency compared to the typical alkaline pH condition (pH 8).[29] After the reaction, solid guanidine HCl was added to a final concentration of 4 M, and the pH adjusted to approximately pH 8 by adding Tris base. Trypsin was then inactivated by reduction with 1 mM DTT at room temperature for 1 h, followed by alkylation with 2.5 mM iodoacetamide at room temperature for 30 min. The resulting ^{16}O- and ^{18}O-labeled peptide solutions were mixed together in equal proportions and desalted using a Vydac C18 reverse phase column. The eluate was dried in a Speed-vac, reconstituted with 0.1% formic acid, and analyzed by LC-MS/MS. In addition to this forward labeling experiment (light exposed OS labeled with ^{18}O, unexposed OS labeled with ^{16}O) we also carried out a reverse labeling experiment (unexposed OS labeled with ^{18}O, light exposed OS labeled with ^{16}O).

LC-MS/MS analysis

Chromatographic separation of the protein digest was performed by an Ultimate 3000 nano-HPLC (Dionex, Germering, Germany) with a trapping pre-column (C18, PepMap100, 300 μm × 5 mm, 5 μm, 100 Å; Dionex, Germering, Germany) followed by a reverse phase column (C18, 75 μm × 150 mm, ^{3} μm, 100 Å; Dionex). Peptides were injected onto the trapping column, which was equilibrated with 0.1% formic acid in water and washed for 5 min with the same solvent at a flow rate of 10 μL/min. After washing, the trapping column was switched in-line with the reverse-phase analytical column and bound peptides eluted using solvents A (0.1% formic acid in water) and B (0.04% formic acid in 80% acetonitrile, 20% water) with a linear gradient of 2% per min, starting with 100% of solvent A at a flow rate of 300 nL/min. The eluted peptides were introduced into a Finnigan linear ion trap Fourier transform (LTQ FT ICR) hybrid mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a 7 T superconducting electromagnet (biological replicates 1 and 2) or LTQ-Orbitrap (Thermo Electron Corp., Bremen, Germany) (biological replicates 3 and 4) mass spectrometer via a silica non-coated PicoTip emitter (FS360-20-10-C12, New Objective Inc., Woburn, MA) at a voltage of 2.2 kV. The capillary temperature was maintained at 200ºC. Full MS spectra were recorded in the FT ICR cell or Orbitrap, and then the tandem mass spectra of the six most intense ions were recorded by the LTQ ion trap at a collision energy of 35 eV, isolation width 2.5 Da, and activation Q at 0.250.

Protein identification

Proteins were identified by comparing all of the experimental peptide MS/MS spectra to the Swiss-Prot (version 57) Rodentia database (25165 proteins) using Mascot database search

software (version 2.1.04, Matrix Science, London, UK). *S*-carbamidomethylation of cysteine was set as a fixed modification while oxidation of methionine (methionine sulfoxide) and C-terminal ^{18}O modification were variable modifications. The mass tolerance for the precursor ion was set to 10 ppm, and for the product ion it was set to 1 Da. Strict trypsin specificity was applied, allowing for one missed cleavage. Only peptides with a minimum score of 20 were considered significant. Scaffold software (Version Scaffold-2_06_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identification. Peptide identifications were accepted if they could be established at an ion score greater than 20, as specified by the Peptide Prophet algorithm.[30] Protein identifications were accepted if they could be established at greater than 95% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.31 Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Calculation of $^{16}O/^{18}O$-peptide ratio

In-house software (Relative Quantification O18.1.2.2) employing a least squares regression algorithm[32] was used for the calculation of $^{16}O/^{18}O$ peptide ratios. This software plots $^{16}O/^{18}O$-peptide intensities of all peptides identified from the same protein, and the slope of the linear regression fit is used as a $^{16}O/^{18}O$ peptide ratio for that protein. Only proteins with $R^2 \geq 0.85$ and a linear regression F-probability greater than 0.85 in at least 1 LC-MS/MS analysis are reported as quantified proteins. Proteins with R^2 values or F-probabilities out of our range were manually investigated for containing possible peptide outliers. An obvious outlier was defined as a peptide whose removal changed the protein R^2 value by more than 0.2 or increased the F-probability to >0.85. If an obvious outlier was detected, it was removed from the peptide list.

The slope of the linear regression fit from all the peptide ratios from all the proteins in the particular sample was also obtained after removing peptides from the following proteins, whose amounts are known to change upon light exposure: arrestin, transducin (Gt_α, Gt_β and Gt_γ), and recoverin. The slope value was then used to normalize the individual protein ratios. This is expected to decrease the influence of experimental error (e.g. pipetting error during sample mixing) on the calculated ratios.

A one sample t-test was used to identify significantly changing proteins. The mean ratio from each biological replicate was tested against the theoretical mean which was set to 1 (no change). Level of significance (α) was set to 0.05. Each protein with a p-value <0.05 and a mean ratio either lower or higher than 2 in at least three biological replicates was considered to be changing its amount after light exposure.

LC-MRM

Isotope dilution tandem mass spectrometry using liquid chromatography multiple reaction monitoring (LC-MRM) was used to verify the results of our proteomic study for rhodopsin and rhodopsin kinase. Reference peptides incorporating stable isotopes at the C-terminal lysine residue were synthesized for rhodopsin (EAAAQQQESATTQ[$^{13}C_6$, $^{15}N_2$]K), and rhodopsin kinase (GITVEEAAPTA[$^{13}C_6$, $^{15}N_2$]K). These tryptic peptides were chosen from among the peptides quantified in our proteomic study and did not contain any known modification sites. Known amounts of these reference peptides were introduced into the tryptic digest of light-exposed and unexposed OS proteins (25 μg) and were used to quantify native peptides derived from rhodopsin and rhodopsin kinase. The digests containing known amounts of the reference peptides were injected onto a C18 reverse phase column (Alltech Altima HP C18, 3um, 1 × 150 mm) equilibrated with 0.02% trifluoroacetic acid (TFA), and then peptides were eluted with a linear gradient of acetonitrile (2%/min) in the presence of 0.1% TFA at a flow rate of

100 μL/min. LC Packings Ultimate (Dionex, Germering, Germany) liquid chromatographic system was used for the chromatography. The peptides eluted from the column were directly introduced into an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Turbo IonSpray ion source and monitored by MRM. The following are the native and reference peptide sequences and the precursor/fragment ions monitored for rhodopsin: EAAAQQQESATTQK [m/z 745.8 (z = 2)/1020.2 (z = 1)] and EAAAQQQESATTQ[$^{13}C_6$,$^{15}N_2$]K [m/z 750.0 (z = 2)/1028.5 (z = 1), and for rhodopsin kinase: GITVEEAAPTAK [m/z 593.8 (z = 2)/816.4 (z = 1)] and GITVEEAAPTA[$^{13}C_6$,$^{15}N_2$]K [m/z 597.8 (z = 2)/824.3 (z = 1)]. Chromatographic peak areas of the native and of the reference peptides were calculated using Analyst Software 1.4.1 (Applied Biosystems), and the area ratios of native/reference peptide were obtained. Then, the ratio for the light-exposed sample was divided by the ratio for the unexposed sample. Two OS preparations were used for the analysis.

Immunocytochemistry

After sacrificing, eyes were removed from animals. Eyecups were prepared by carefully removing the cornea and lens. Eyes were dissected under light for light-exposed samples and under dim red light illumination for unexposed control samples. The eyecups were fixed with 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) for at least 6 hours. Then, the paraformaldehyde solution was exchanged in four concentration steps (5%, 10%, 15% sucrose) for 20% sucrose in 100 mM phosphate buffer (pH 7.4). Eyecups were then incubated overnight in a mixture of 20% sucrose phosphate buffer and Optimal Cutting Temperature (OCT) compound at a 2:1 ratio. Eyecups were frozen in isopentane cooled by liquid nitrogen and sectioned at 12 μm using a Leica CM 1850 Cryostat (Leica Microsystems Inc., Bannockburn, IL). For immunofluorescence, eyecup sections were first blocked for nonspecific labeling by incubating in 1.5% normal goat serum in PBST buffer (136 mM NaCl, 11.4 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) for 15 min at room temperature. Sections were then incubated with anti-rhodopsin kinase antibody[33] in PBST overnight at 4°C. Sections were rinsed in PBST and incubated with indocarbocyanine (Cy3)-conjugated secondary antibodies. Sections were then rinsed in PBST and mounted in 50 μl of 2% 1,4-diazabicyclo-2,2,2-octane in 90% glycerol to slow photobleaching. Sections were analyzed under a Leica DM6000 B microscope (Leica Microsystems Inc.) equipped with a RETIGA EXi CCD camera (QImaging, Burnaby, BC. Canada).

Results

Comparative proteomic study on intense light-exposed OS

Proteins from four biological replicates of light-exposed and unexposed OS were subjected to proteolytic ^{18}O labeling and analyzed by LC-MS/MS. All replicates were subjected to forward (F1-F4 experiment) and reverse (R1-R4 experiment) ^{18}O labeling. A total of 171 proteins were identified of which 58% (100 proteins) were classified as membrane proteins (Supplemental Table 1), demonstrating that the method we used was effective at identifying membrane proteins. Out of 171 proteins identified, 98 passed our statistical criteria (R^2>0.85 and F-probability>0.85) for quantification (Supplemental Table 2). Figure 1 shows the plots of the protein ratios obtained in the forward experiment against those for the same proteins in the reverse experiment (Fig. 1a: F1 *vs*. R1 experiment, Fig 1b: F2 *vs.* R2 experiment, Fig 1c: F3 *vs*. R3, and Fig 1d F4 *vs* R4). Linear regression analyses on the plots were performed and the regression line (bold line) as well as the confidence interval lines with α = 0.01 (dotted lines) are shown in the figure. The theoretical line y=1 (represented by the diagonal of each panel) is within the confidence interval lines for all replicates, demonstrating that the results between the forward and reverse labeling experiment with the same biological sample were consistent.

The quantities of 11 proteins differed by at least 2-fold between light-exposed and unexposed OS samples in at least three biological replicates. Proteins altered in only one or two replicates were eliminated from consideration. Among the 11 proteins, 8 were proteins involved in phototransduction: arrestin, guanine nucleotide-binding protein Gtα, Gtβ and μ Gtγ, recoverin, rhodopsin kinase, and rod cGMP-specific 3′,5′-cyclic phosphodiesterase α and β (Table 1). The quantities of several other phototransduction proteins were not altered by light exposure (Table 1). Those proteins were cGMP-gated cation channel α, guanine nucleotide-binding protein β-5, guanylyl cyclase GC-E, regulator of G-protein signaling 9 and rhodopsin. Besides phototransduction proteins, we found three non-phototransduction proteins whose amounts increased more than 2-fold in light-exposed samples (Table 2). They include ubiquitin, heat shock protein HSP 90-alpha and heat shock cognate 71 kDa protein.

A total of 8 glycolytic enzymes were quantified and found to be unchanged by light (Table 3). Major OS integral membrane proteins, such as rod outer segment membrane protein 1 (ROM1) and retinal-specific ATP-binding cassette transporter, were also found to be unchanged (Supplemental Table 2).

LC-MRM analysis

Rhodopsin kinase and rhodopsin were quantified by LC-MRM in two biological replicates to verify our proteomic results. Rhodopsin was selected to ensure that no significant photoreceptor degeneration was induced during light exposure. Rhodopsin kinase was selected because the decrease in protein amount upon light exposure did not appear to be a common adaptive change to light (see the Discussion), and therefore, was unique among the identified changes. The first LC-MRM verification experiment was carried out with the same sample (biological replicate 1) that was used for the proteomic study described above. In this sample, rhodopsin levels did not change upon light exposure, while the amount of rhodopsin kinase decreased 2-fold after light exposure (Table 4). In our proteomics study the light exposed/unexposed ratios of the rhodopsin peptide, EAAAQQQESATTQK, and the rhodopsin kinase peptide, GITVEEAAPTAK, between F1 and R1 was 1.26 and 0.42, respectively. Thus, the results were consistent with the proteomic results. In the second LC-MRM experiment, a new preparation of rat OS was analyzed. The light exposed/unexposed ratios of rhodopsin and rhodopsin kinase in the newly prepared OS were 0.75 and 0.26, respectively. Thus, the LC-MRM results confirm that the amount of rhodopsin was not altered by 8 hr light exposure, while rhodopsin kinase in the OS decreased more than 2-fold.

Immunocytochemistry

We investigated whether the reduction in the rhodopsin kinase quantified by MS analysis was due to its translocation from OS to IS, or due to a reduction of the absolute level in photoreceptors. To discriminate between those possibilities, localization of rhodopsin kinase was examined by immunofluorescence microscopy. Cryosections of eye cups were prepared from the rats exposed (Figure 2a) and unexposed (Figure 2b) to light, under identical conditions used for OS sample preparation. Rhodopsin kinase was localized exclusively in the rod and cone photoreceptor OS, regardless of light conditions. This indicates that the absolute quantity of rhodopsin kinase decreased in the photoreceptors of light exposed rats.

Discussion

Among the 8 phototransduction proteins found to be altered by light, 7 proteins reduce or quench phototransduction signaling upon intense light exposure, and therefore their changes appear to be adaptive changes to light. For instance, the amount of proteins involved in the activation steps of the phototransduction cascade (guanine nucleotide-binding protein Gtα, Gtβ and Gtγ, and rod cGMP-specific 3′,5′-cyclic phosphodiesterase α and β) decreased 3.2- to

6.7-fold. In contrast, the level of arrestin, which is involved in quenching the signaling cascade, increased greater than 10-fold. The decrease in recoverin (5.6 fold) can also be considered adaptive, because it reduces the inhibitory constraint that recoverin imposes on rhodopsin kinase.[34] Proteins involved in the activation and deactivation of phototransduction cascade are depicted in Figure 3a and b, respectively.

One unusual protein was rhodopsin kinase, whose amount decreased 2-fold after light exposure. This result was verified by LC-MRM analysis on two OS preparations, which showed a 2.0- and 3.8-fold reduction of this protein after light exposure (Table 4). Rhodopsin kinase helps to quench the light-induced transduction signaling cascade by phosphorylating rhodopsin; therefore, an increased amount of this protein would be expected to deactivate the cascade. Indeed, mice lacking rhodopsin kinase are much more susceptible to photoreceptor cell damage from light compared to wild-type animals, which is due to prolonged activation of rhodopsin.[6, 17] Furthermore, hemizygous rhodopsin kinase knockout mice express ~ 2 fold less enzyme than wild type, and have a slower dark recovery rate to dim light flashes.[17] Therefore, it is reasonable to believe that a 2-fold reduction of rhodopsin kinase can provide a physiologically relevant change in the rod photoresponse. Our finding suggests that the loss of rhodopsin kinase might be a contributing factor to light-induced photoreceptor degeneration by prolonging the lifetime of activated rhodopsin.

Since the total rhodopsin kinase amount in rod OS appears to be reduced by light, we propose two alternate possibilities: 1) the degradation of rhodopsin kinase was accelerated upon light exposure, or 2) rhodopsin kinase is continuously degraded, and its synthesis was slowed by light exposure. Previous studies suggested that rhodopsin kinase does not change its distribution upon light exposure,[34] a conclusion mainly drawn from Western blotting analyses. Although Western blotting is useful to study relative protein quantities among different subcellular compartments in the photoreceptors, it does not quantify proteins at the same accuracy as LC-MRM. In contrast to the previous reports, our LC-MRM quantification clearly indicates that there is a mechanism to reduce rhodopsin kinase amount in OS upon light exposure, without changing the overall distribution in the photoreceptor cells.

Among the eight phototransduction proteins whose amounts were altered by light, four proteins, guanine nucleotide-binding protein Gtα, Gtβ and Gtγ,[35, 36] and recoverin,[34] have been reported to translocate from OS to other compartments in photoreceptor cells upon light exposure, while arrestin is known to translocate in the opposite direction.[36, 37] In our proteomic study, the amounts of guanine nucleotide-binding protein Gtα, Gtβ and Gtγ, and recoverin were reduced 5.3-, 5.9-, 6.7-, and 5.6-fold, respectively, while arrestin increased more than 10-fold after light exposure (Table 1), consistent with previous findings. We also found that the amounts of rod cGMP-specific 3′,5′-cyclic phosphodiesterase α and β were reduced 3.2- and 3.4-fold, respectively, after light exposure (Table 1). However, a previous study did not observe light-driven translocation of these proteins.[36] This discrepancy may be due to the differences in light treatment conditions. Whereas we used intense (1200 lux) and prolonged (8 h) light treatments, mild (250 lux) and short (1 h) light treatments were used in the previous report. Further studies are necessary to clarify whether the reduced amounts of these proteins in the OS after light exposure are due to translocation of these proteins out of the OS or are due to other mechanisms.

We found that the levels of heat shock protein HSP 90-alpha, heat shock cognate 71 kDa protein and ubiquitin are elevated more than 2-fold after light exposure (Table 2). Heat shock proteins are molecular chaperones and known to be induced under various stress conditions.[38] Their major function is to assist in the folding of nascent polypeptides or denatured proteins that result from various cellular stresses.[39] Thus, our results imply that misfolded proteins accumulate in the OS upon intense light exposure and that the induction of heat shock proteins

is to protect cells against light-induced oxidative stress. Ubiquitin is a protein modifier which can be covalently attached to proteins that marks the proteins for degradation by the proteasome.[40] The existence of ubiquitin-dependent proteolysis pathways in OS have been reported.[41] Therefore, the elevated ubiquitin level may be suggesting a higher activity of ubiquitin-proteasome pathways in intense light exposed OS.

A total of 8 glycolytic enzymes were quantified and found to be unchanged by light (Table 3), suggesting that no significant change occurred in glycolysis. No significant changes in major OS integral membrane proteins, including rhodopsin, rod outer segment membrane protein 1 (ROM1), cGMP-gated cation channel alpha-1 and retinal-specific ATP-binding cassette transporter, were found (Supplemental Table 2). The fact that the levels of soluble glycolytic enzymes and major OS integral membrane proteins were unchanged after light indicates that light-induced membrane damage, that may have caused a leaky OS, was not significant.

This is the first comparative proteomic study of intense light-induced changes in OS proteins and provides a foundation for further proteomic research. Our study led to the unexpected discovery of changes in the quantity of rhodopsin kinase during intense light exposure, a finding that could improve our understanding of light-induced photoreceptor apoptosis. It is currently unknown how light regulates the quantities of specific proteins in photoreceptor cells. In our analysis, another isoprenylated protein, rod cGMP-specific 3′,5′-cyclic phosphodiesterase α and β were down-regulated similar to rhodopsin kinase (Table 1). An intriguing hypothesis is that rod cGMP-specific 3′,5′-cyclic phosphodiesterase α and β and rhodopsin kinase are degraded specifically by a common pathway. Recently, a prenyl binding protein, PrBP/delta was demonstrated to be involved in specific trafficking of rhodopsin kinase and PDE6 to the photoreceptor outer segment.[42] Similarly, there could be a specific mechanism to traffic the isoprenylated proteins to the proteolytic machinery in photoreceptors. This novel protein quality/quantity control pathway warrants further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Gaurav S. J. B. Rana for assistance in statistical analysis and Kerrie Lodowski for helpful discussions and editorial advice. The authors are grateful to Krzysztof Palczewski for generously providing us with anti-rhodopsin kinase monoclonal antibody G8. This study was supported by an NIH grant P30EY-11373 (Visual Sciences Research Center of Case Western Reserve University) and funds from Case Western Reserve University and the Cleveland Foundation (MM), and by the Ohio Lions Eye Research Foundation and M. Petticrew of Springfield OH (DTO).

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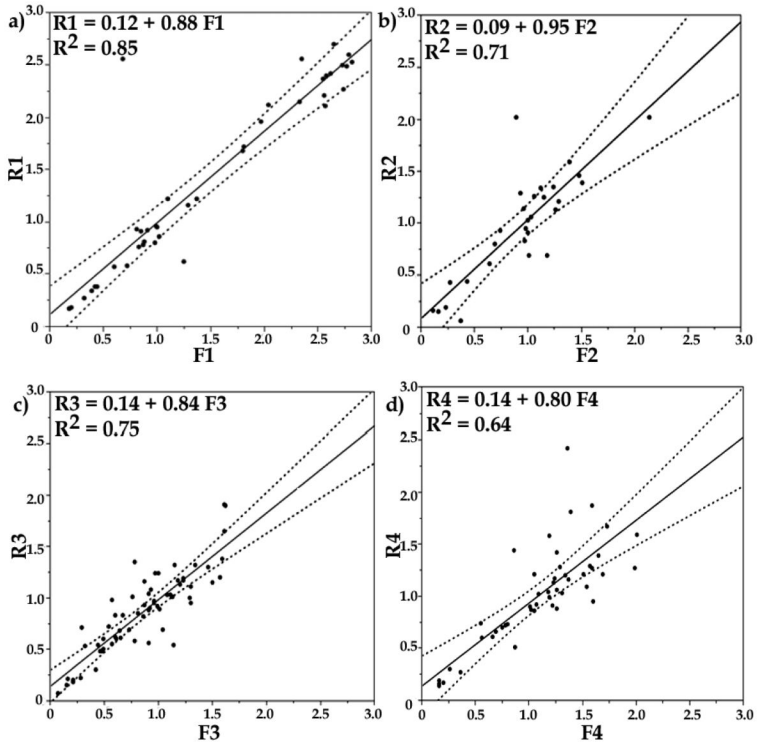

Figure 1.

Linear regression analysis of protein ratios in forward and reverse ^{18}O labeling experiments. A simple linear regression analysis of individual protein ratios in the R1 experiment as a function of those in the F1 experiment (a), R2 as a function of F2 (b), R3 as a function of F3 (c), and R4 as a function of F4 (d) were conducted. The regression line (bold line) and the confidence interval lines with $\alpha = 0.01$ (dotted lines) are shown. The equations and R^2 values for the regression lines are shown in left top corner of each panel. Ratios greater than 3.0 were excluded from the analysis.

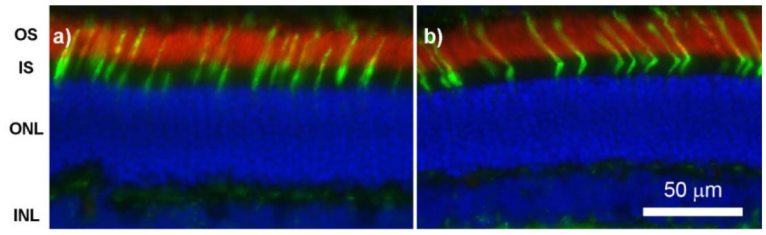

Figure 2.

Immunocytochemical analysis of rhodopsin kinase. Representative immunofluorescence images from rats unexposed to light (a) and exposed to light for 8 hr (b) are shown. Rhodopsin kinase (red) was detectable only in the OS. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) and cone photoreceptors with fluorescein-conjugated peanut agglutinin (green). OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; INL, inner nuclear layer. Three light exposed and three unexposed rats were analyzed.

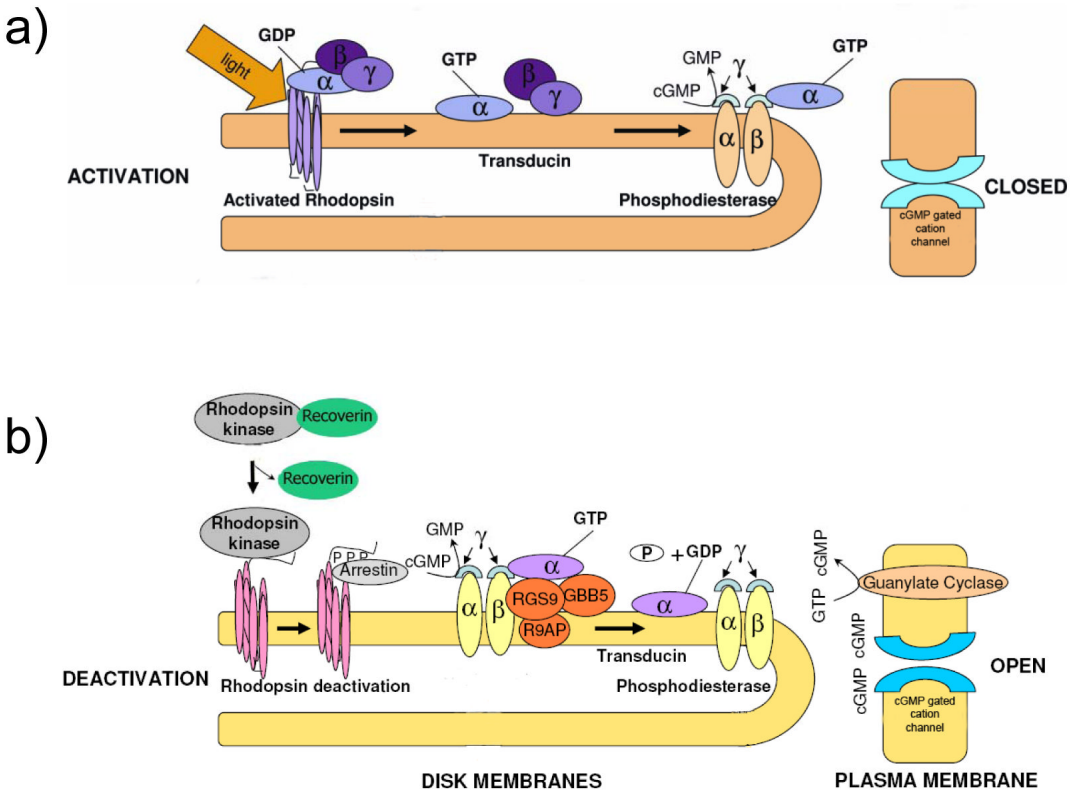

Figure 3.

Molecular steps in activation (a) and deactivation (b) of the phototransduction cascade. The activation cascade is initiated upon photon absorption by rhodopsin. Absorption of a photon by rhodopsin causes a series of conformational changes in the protein leading to an activated state of rhodopsin. The activated rhodopsin binds a heterotrimeric protein transducin and allows for exchange of GDP nucleotide for GTP in the α subunit of transducin. The GTP bound α subunit dissociates from the β and γ subunits of transducin and activates phosphodiesterase that catalyzes hydrolysis of cGMP. In response to a decreased concentration of cGMP, cGMP gated cation channels in the plasma membrane close, resulting in decreased calcium levels which cause the photoreceptor cell to becomes hyperpolarized (a). Recoverin is normally bound to rhodopsin kinase when the calcium concentration is high. However, during phototransduction the calcium levels fall, resulting in the release of rhodopsin kinase that leads to initiation of the deactivation cascade of rhodopsin. The activated rhodopsin is first phosphorylated by rhodopsin kinase. The phosphorylated rhodopsin allows arrestin to bind, which stearically interferes with the binding of the α subunit of transducin. In addition to deactivating rhodopsin, transducin mediated signaling could be quenched. The complex of G-protein beta 5 (GBB5), regulator of signaling 9 (RGS9) and regulator of signaling 9 anchor protein (R9AP) binds to the transducin α subunit-phosphodiesterase complex. This stimulates the GTPase activity of transducin α subunit and results in dissociation of the transducin α subunit from phosphodiesterase, therefore slowing down the hydrolysis of cGMP, allowing the cGMP channels to open (b). Adapted from Burns and Arshavsky.[43]

Table 1

Proteins involved in phototransduction

SwissProt Accession #	SwissProt ID	Protein Name	MW (Da)	Average protein ratio[a]	Standard deviation	P-value	# of unique peptides quantified
Proteins found in greater amount in light exposed OS							
P15887	ARRS_RAT	Arrestin	45206	>10[b]			22
Proteins found in lesser amount in light exposed OS							
P20612	GNAT1_MOUSE[c]	Guanine nucleotide-binding protein G(t) alpha-1	40397	0.19	0.01	<0.001	16
P54311	GBB1_RAT	Guanine nucleotide-binding protein G(I)/G(S)/G(T)beta-1	38151	0.17	0.01	<0.001	17
Q61012	GBG1_MOUSE	Guanine nucleotide-binding protein G(T) gamma-T1	8693	0.15	0.02	<0.001	9
P34057	RECO_MOUSE	Recoverin	23449	0.18	0.11	0.006	6
Q63651	RK_RAT	Rhodopsin kinase	64298	0.51	0.10	0.002	15
P27664	PDE6A_MOUSE	Rod cGMP-specific 3′,5′-cyclic phosphodiesterase alpha	100437	0.31	0.06	<0.001	18
P23440	PDE6B_MOUSE	Rod cGMP-specific 3′,5′-cyclic phosphodiesterase beta	99693	0.29	0.09	0.001	8
Proteins unchanged							
Q62927	CNGA1_RAT	cGMP-gated cation channel alpha-1	79577	0.99	0.06	0.654	8
P62882	GBB5_RAT	Guanine nucleotide-binding protein beta-5	39505	0.99	0.12	0.928	5
P51840	GUC2E_RAT	Guanylyl cyclase GC-E	121750	0.98	0.17	0.834	18
P49805	RGS9_RAT	Regulator of G-protein signaling 9	77653	0.94	0.23	0.629	6
P51489	OPSD_RAT	Rhodopsin	39586	1.14	0.67	0.335	6

[a] Calculated using protein ratios obtained from four biological replicates. The ratios are light exposed/unexposed.

[b] Exact value is not shown, because this proteolytic method cannot provide accurate value when the protein ratio exceeds greater than 10. Exact value can be seen in Supplemental Table 2.

[c] Mouse SwissProt accession numbers and IDs are used for proteins for which rat sequences have not been reviewed in SwissProt database version 57.0

Table 2

Non-phototransduction proteins found to be altered

SwissProt Accession #	SwissProt ID	Protein Name	MW (Da)	Average protein ratio[a]	Standard deviation	P-value	# of unique peptides quantified
P62989	UBIQ_RAT	Ubiquitin	8560	3.97	1.04	0.011	4
P82995	HS90A_RAT	Heat shock protein HSP 90-alpha	85134	4.13	1.13	0.041	11
P63018	HSP7C_RAT	Heat shock cognate 71 kDa protein	70989	2.50	0.90	0.045	14

[a]Calculated using protein ratios obtained from the eight proteomic experiments on four biological replicates. The ratios are light exposed/unexposed.

Table 3

Glycolytic enzymes

SwissProt Accession #	SwissProt ID	Protein Name	MW (Da)	Average protein ratio[a]	Standard deviation	P-value	# of unique peptides quantified
P05065	ALDOA_RAT	Fructose biphosphate aldolase alpha	39783	0.78	0.11	0.028	13
P09117	ALDOC_RAT	Fructose biphosphate aldolase gamma	39658	1.05	0.48	0.837	12
P04764	ENOA_RAT	Alpha enolase	47440	0.71	0.15	0.030	15
P07323	ENOG_RAT	Gamma enolase	47510	1.00	0.27	0.980	4
P04797	G3P_RAT	Glyceraldehyde-3-phosphate dehydrogenase	36090	1.06	0.12	0.351	10
P11980	KPYM_RAT	Pyruvate kinase isozymes M1/M2	58294	0.82	0.14	0.085	16
P25113	PGAM1_RAT	Phosphoglycerate mutase 1	28928	0.65	0.34	0.21	6
P16617	PGK1_RAT	Phosphoglycerate kinase	44909	0.81	0.11	0.106	9
P48500	TPIS_RAT	Triose phosphate isomerase	27345	1.00	0.42	0.986	7

[a]Calculated using protein ratios obtained from four biological replicates. The ratios are light exposed/unexposed.

J Proteome Res. Author manuscript; available in PMC 2011 February 5.

Table 4

Rhodopsin kinase and rhodopsin ratios analyzed by LC-MRM

	1st replicate	2nd replicate
Protein	**[a]Ratio**	**[a]Ratio**
rhodopsin kinase	0.52	0.26
rhodopsin	1.09	0.75

[a]Mean ratio light exposed/unexposed from two LC-MRM injections.