Light Induced Protein Modifications and Lipid Oxidation Products in Rat Retina

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Abstract

Purpose: To better understand the mechanisms of oxidative damage in retinal pathology, we have sought the identity of lipid oxidation products and protein adducts in rat retina after *in vivo* exposure to damaging light.

Methods: Albino rats maintained in a dark environment for 2 months were exposed to intense green light (1500 lux) for 1 or 4 hours and sacrificed immediately following light treatment. Retinas were isolated and immediately protected with antioxidants. Lipids were extracted with chloroform/methanol and analyzed by LC MS. Proteins were extracted with SDS-PAGE sample buffer and analyzed by Western blotting.

Results: Lipid oxidation products in rat retina from docosahexaenoyl phosphatidylcholine (DHA-PC), arachidonoyl (AA)-PC, and linoleyl (LA)-PC were more abundant after 4 h of light exposure than after 1h or no light. Anti-carboxyethylpyrrole, anti-argpyrimidine and anti-nitrotyrosine immunoreactivities were significantly greater after 4h light exposure compared with control animals maintained in the dark. Anti-opsin immunoreactivity was also significantly greater after light treatment. The 2-D analysis of rat retina revealed that CEP and argpyramidine modifications on proteins might be produced at the same location. Using western analysis, Mass spectrometric analysis of 2D-gel spots identified several putative Argpyrimidine and CEP modified proteins

Conclusions: Current results are consistent with our recent observation that light modulates protein nitration in rat retina (2002 *Mol. & Cell. Proteomics* **1**, 293). Intense light also generates lipid oxidation products in rat retina *in vivo* that result in oxidative protein modifications such as carboxyethylpyrrole from DHA containing lipids. Argpyrimidine, derived from methylglyoxal, appears to be another protein modification induced by light. The apparent increase in opsin after light may be due to modifications that increase the solubility and extractability of this integral membrane protein. These findings justify further consideration of lipid oxidation products and protein modifications as mediators in the light-induced biochemical sequela leading to photoreceptor cell death.

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Introduction

The molecular mechanisms causing retinal damage from intense light are poorly understood but appear to involve oxidative stress since antioxidants can provide protection (Organisciak et al., 1999). Damaging light is thought to produce reactive oxygen species but the identity of such species and the pathways of oxidative damage remain unknown. Our recent proteomic analyses of rat retina after 1500 lux light exposure for 3h have shown that light modulates tyrosine nitration (Miyagi et al., 2002), that crystallin 2D gel components increase 2-3 fold relative to unexposed retinas and that crystallin immunoreactivity increases in rod outer segments and RPE (Sakaguchi et al., 2003). Age-related macular degeneration (AMD) also involves oxidative damage and crystallin appears to be more common in drusen from AMD donor eyes than from normal human eyes (Crabb et al., 2002). Carboxyethylpyrrole (CEP) adducts, a protein modification uniquely derived from oxidation of docosahexaenoate-containing lipids, are also more abundant in Bruch's membrane/RPE/choroid from AMD donor eyes (Crabb et al., 2002). As an approach to better understanding mechanism of light damage, here we probe rat retina for changes in lipid oxidation products, oxidative protein modifications and retinal proteins following intense light exposure. The results support additional similarities with AMD.

Methods

Animal Procedures and Retinal Preparations: Albino Sprague-Dawley rats were maintained in a dark environment for 2 months prior to exposure to intense green light at 1500 lux for 1 and 4 hours. Animals were scarified immediately following light exposure, retinas excised within 2 min of death, rinsed in PBS containing 2 mM DTPA and 100 µM butylated hydroxytoluene (BHT) and frozen in liquid nitrogen. The following day retinas were thawed and lipids first extracted with chloroform/methanol and dried under nitrogen then protein extracted with Laemmli SDS sample buffer under dim red illumination.

Identification of Lipid Oxidation Products: The lipid extracts were resuspended in 50% methanol containing 0.1% formic acid and analyzed using a Quatro II triple quadrupole electrospray mass spectrometer (Micromass) interfaced with an HP 1100 HPLC, a Vydac C18 column (2.1 x 150 mm) at a flow rate of 0.2 ml/min. Tandem mass spectrometry was performed on line in positive ion mode with multiple reaction monitoring (MRM) (cone potential of 60 eV/collision energy 20-25 eV). The MRM transitions used to detect oxidized phospholipids were the mass to charge ratio for the molecular cation [MH] + and the daughter ion *m/z* 184, the phosphatidylcholine group (i.e., [MH]+→ *m/z* 184). Synthetic phospholipid oxidation products were used as authentic standards in LC MS/MS as described elsewhere (Podrez et al., 2002).

2D-Gel Electrophoresis and Western Analysis: Retinal protein was precipitated using two volumes of acetone, redissolved in solution 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 2% IPG-ampholytes pH 3-10, 1% DTT, and subjected to 2D PAGE. First dimension IEF was performed with the Pharmacia IPGphor system and non-linear pH 3-10 immobilized pH gradients (7 cm). Second dimension electrophoresis was according to Laemmli on 10 % acrylamide gels with a 4% stacking gel (10 x 8 cm). Image analysis utilized PDQuest software (Biorad). Protein was quantified using the Bradford assay. One and two dimensional electrophoresis and Western blotting methods were as described (West et al., 2001; Miyagi et al., 2002). Antibodies utilized in Western blots include anti-nitrotyrosine mAb (Upstate Biotechnology, Lake Placid, NY), anti-CEP mAb (Crabb et al., 2002), anti-Argpyrimidine mAb (Padayatti et al., 2001), anti-RPE 65 mAb (gift from J Saari), rat anti-photoreceptor all-*trans*-retinol dehydrogenase pAb (Rattner et al., 2000) and anti-rhodopsin mAb B6-30N (Adamus et al., 1991) and rabbit anti-CRALBP pAb (Crabb et al., 1991).

Protein Identification.: Gel bands/spots were excised, digested with trypsin and proteins identified by LC MS/MS using a CapLC system and a quadrupole time-of-flight mass spectrometer, ProteinLynxTM Global Server and MassLynxTM software (Micromass), and the Swiss-Protein and NCBI protein sequence databases (Crabb et al., 2002).

Results

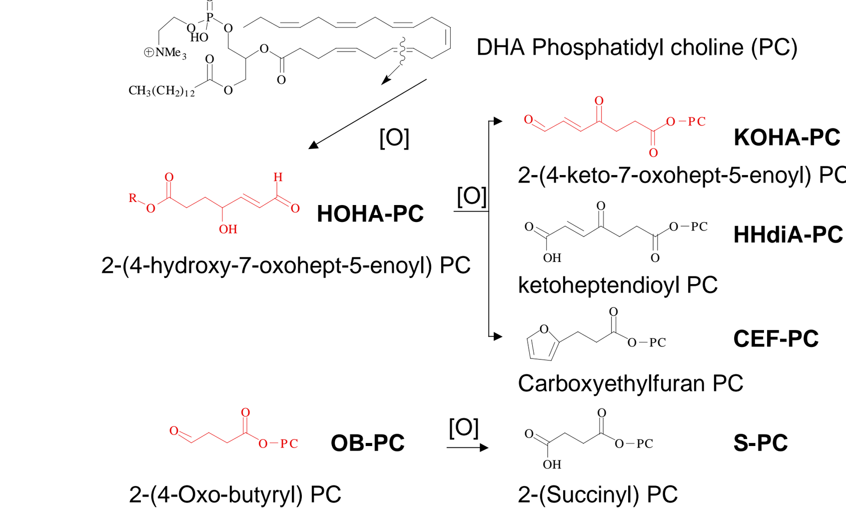

Figure 1. Oxidation of Docosahexaenoyl Phosphatidyl Choline (DHA-PC). The aldehydic lipid fragments generated from oxidation of DHA-PC are highly reactive compounds that may modify proteins.

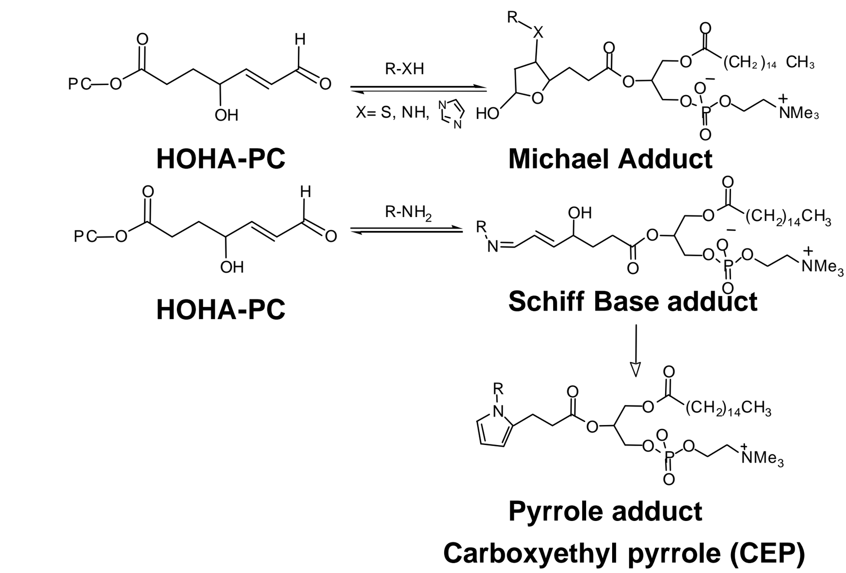

Figure 2. Protein Adducts Generated from the Oxidation of DHA-PC. HOHA-PC possesses γ-hydroxy-αβ-unsaturated aldehyde functionality, which may react with amino acids to form Michael, Schiff base, and pyrrole protein adducts. Previous studies suggests that lysine-based Michael and Schiff base adducts are reversible, while Pyrrole adducts are stable.

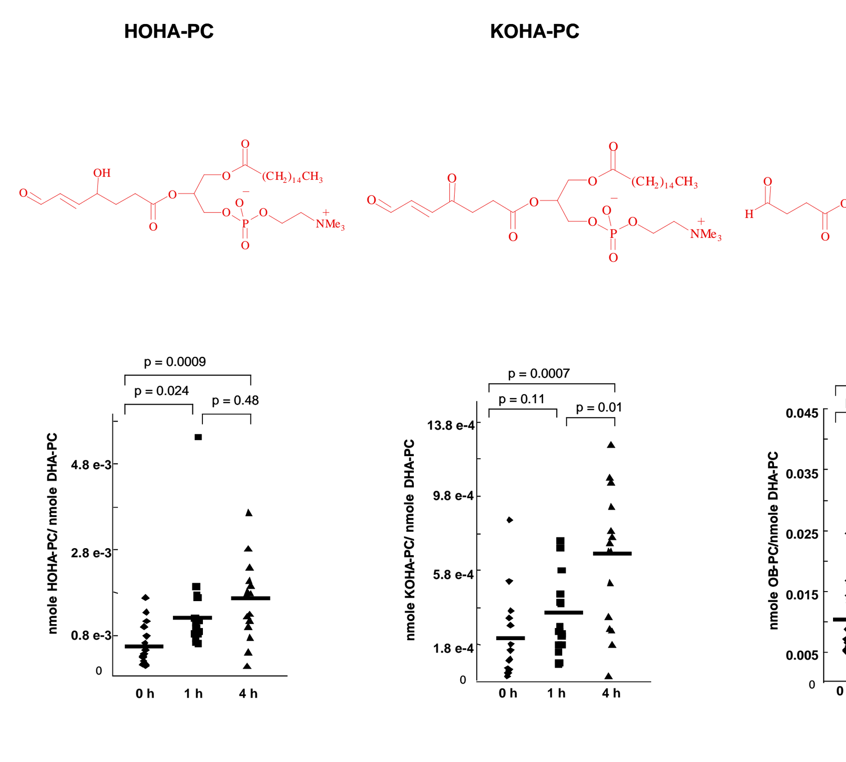

Figure 3: DHA-PC Lipid Oxidation Products Increase with Light Exposure. Lipids were extracted from rat retina after light exposure (1500 lux) for 0 h, 1h or 4h and analyzed by LC MS/MS [n=8 rats (16 retinas) per time point].

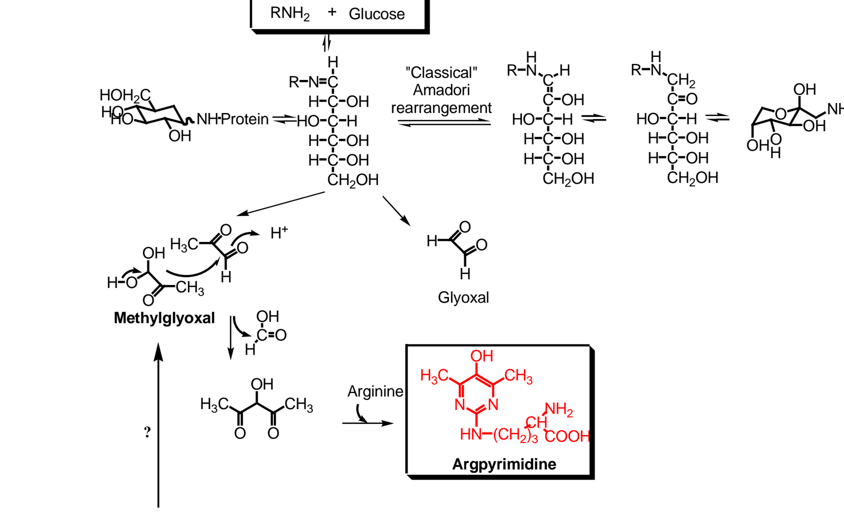

Figure 4. Formation of Argpyrimidine from Methyl Glyoxal. Argpyrimidine is an advanced glycation end product from the reaction of methyl glyoxal and arginine. Methyl glyoxal is also suspected of being a lipid oxidation product.

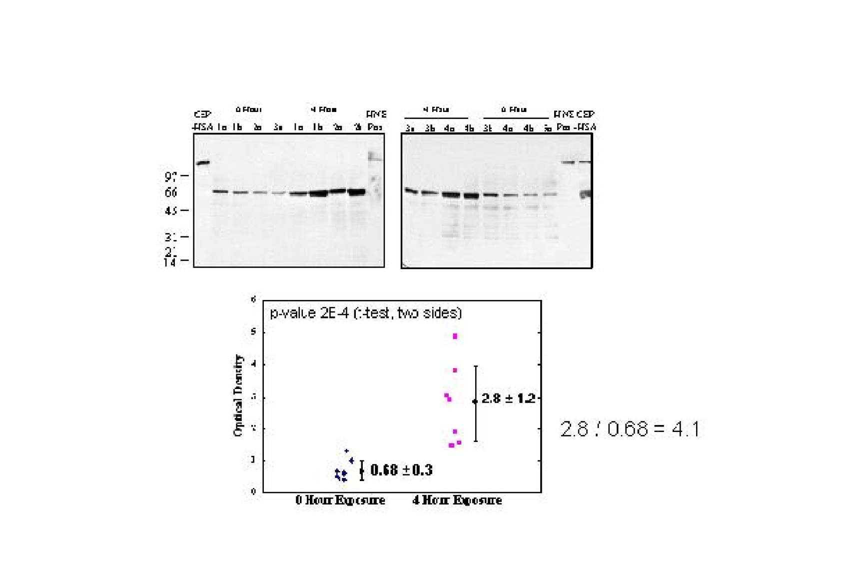

Figure 5: Carboxyethyl pyrrole (CEP) Immunoreactivity Increases with Light Exposure. Western analysis of rat retina with anti-CEP mAb before (0 h) and after (4 h) *in vivo* light exposure (1500 lux). CEP-HSA was used as the positive control.

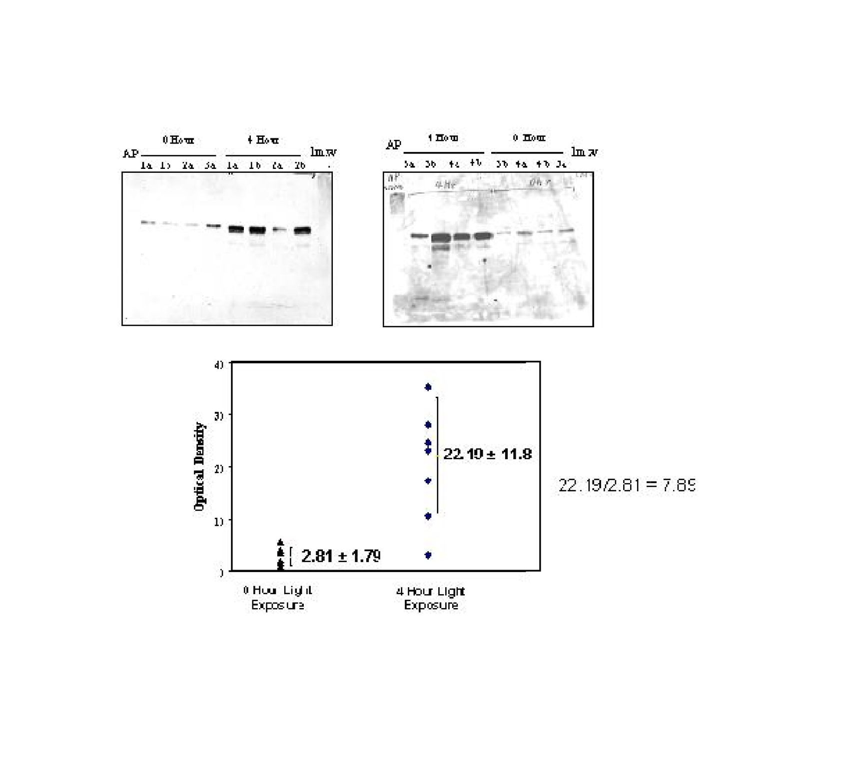

Figure 6: Argpyrimidine (AP) immunoreactivity Increases with Light Exposure. Western analysis of rat retina with anti-AP mAb before (0 h) and after (4 h) *in vivo* light exposure (1500 lux). AP-crystallin was used as the positive control.

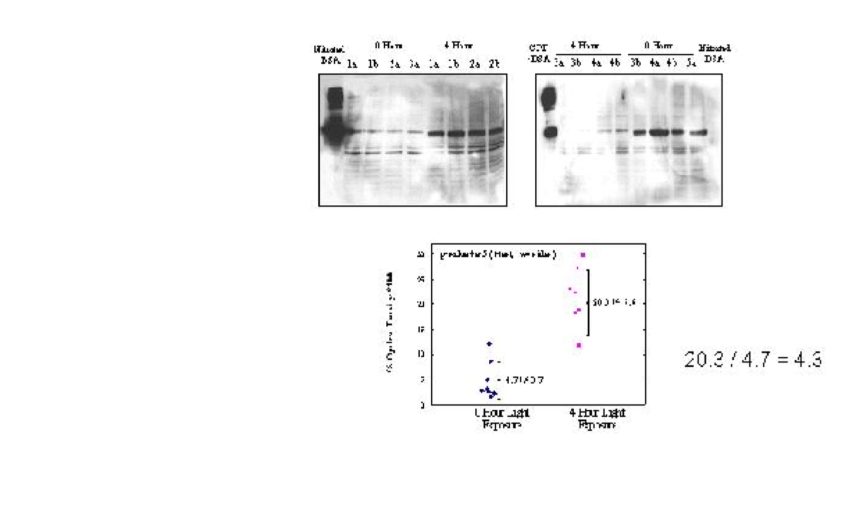

Figure 7: Nitrotyrosine (nY) immunoreactivity Increases with Light Exposure. Western analysis of rat retina with anti-nY mAb before (0 h) and after (4 h) *in vivo* light exposure (1500 lux). Nitrated BSA was used as the positive control.

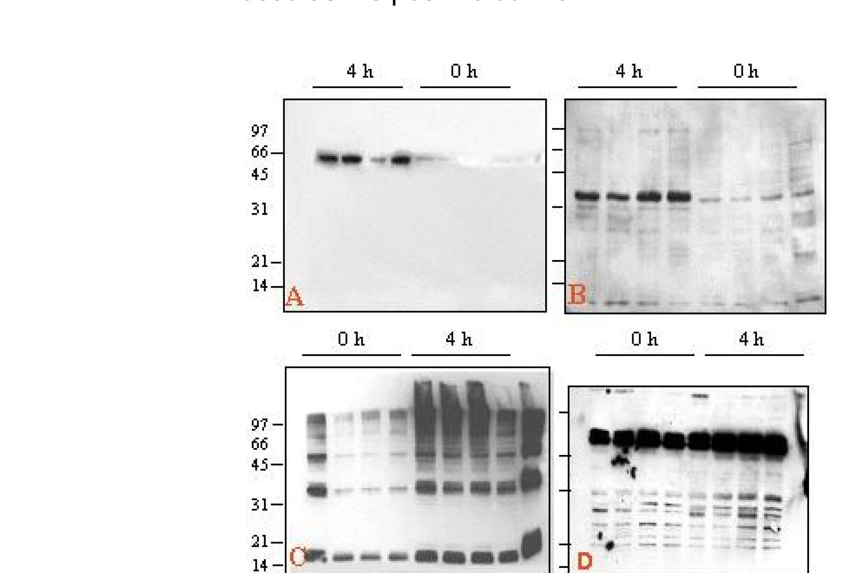

Figure 8: Select Protein Immunoreactivity Increases with Light Exposure. Western analysis of rat retina before (0 h) and after (4 h) *in vivo* light exposure (1500 lux) using (A) anti-RPE 65, (B) anti-photoreceptor all-*trans*-retinol dehydrogenase (prRDH), (C) anti-rhodopsin (D) anti-CRALBP. Positive control used were bovine rod outer segments, ROS. Results are shown from 4 animals at each time point; similar results were obtained from all 8 rats. The immunoreactivity of photoreceptor proteins RPE 65, prRDH, and rhodopsin increased following light exposure but CRALBP immunoreactivity (in Müller cells) and actin (not shown) did not change.

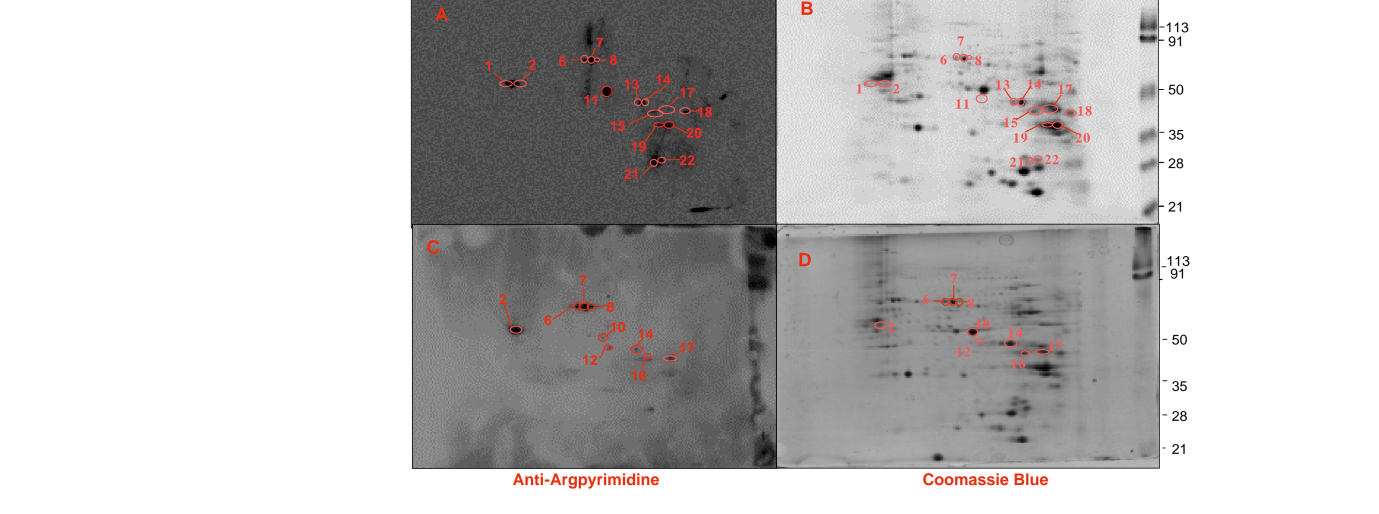

Figure 9. Rat Retinal Proteins after Light Exposure. 2D-PAGE analysis of rat retinal homogenates (50 mg protein/gel) was performed following *in vivo* light exposure. Gels were partially blotted to PVDF membranes for Western analysis with (B) anti-CEPmAb or (D) anti-argpyrimidine mAb. Immunoreactive spots were excised from Coomassie blue stained gels (A, C) for protein identification (Table 1).

Table I: Retinal Proteins in Immunoreactive Gel Spots‡

Spot No		Proteins	No. Peptide Matches	Molecular Mass (kD) / pI Observed	Molecular Mass (kD) / pI Calculated	Swiss Protein Accession No.
Carboxyethylpyrrole Immunoreactive (Gel B)						
1		Tubulin, beta	11	45 / 4.5	50 / 4.79	P04691
2		Tubulin, beta	12	45 / 4.8		P04691
		Vimentin	6		54 / 5.06	P31000
6	*	Serum albumin	4	66 / 6	69 / 6.09	P02770
7	*	Serum albumin	8	66 / 6.1		P02770
8	*	Serum albumin	2	66 / 6.2		P02770
13		Glutamine synthetase	6	42 / 6.9	42 / 6.64	P09606
14		Glutamine synthetase	2	42 / 7.2		P09606
15		Fructose-bisphosphate aldolase A	10	39 / 7.5	39 / 8.4	P05065
17		Aspartate aminotransferase, cytoplasmic	8	39 / 8	46 / 6.3	P13221
		Fructose-bisphosphate aldolase A	7	39 / 8	39 / 8.4	P05065
		Fructose-bisphosphate aldolase C	3	39 / 8	39 / 6.79	P09117
18		Aspartate aminotransferase, mitochondrial	11		47 / 9.13	P00507
19		Glyceraldehyde 3-phosphate dehydrogenase	8		36 / 8.44	P04797
	*	Malate dehydrogenase, mitochondrial	2	35 / 7.5	36 / 8.92	P04636
20		Glyceraldehyde 3-phosphate dehydrogenase	7	35 / 8.2	36 / 8.44	P04797
21		Glyceraldehyde 3-phosphate dehydrogenase	3	25 / 7		P04797
Argpyrimidine Immunoreactive (Gel D)						
2		Vimentin	5	50 / 4.8	54 / 4.8	P31000
		Tubulin, beta	2		50 / 4.8	P04691
6	*	Serum albumin	5	66 / 6.1	69 / 6.09	P02770
	*	Dihydropyrimidinase related protein-2	4		62 / 5.95	P47942
	*	Dihydropyrimidinase related protein-1	1		62 / 6.64	Q62950
7	*	Serum albumin	15	66 / 6.2	69 / 6.09	P02770
8	*	Serum albumin	6	66 / 6.4		P02770
10		Enolase, alpha	13	45 / 6.6	47 / 5.84	P04764
		Enolase, beta	3		47 / 7.74	P15429
		Enolase, gamma	2		47 / 5.03	P07323
12		Adenosylhomocysteinase	5	43 / 6.6	47 / 6.08	P10760
14		Glutamine synthetase	6	43 / 7.3	42 / 6.64	P09606
16		Fructose-bisphosphate aldolase C	7	40 / 7.5	39 / 6.79	P09117
17		Fructose-bisphosphate aldolase C	1	40 / 8		P09117
		Fructose-bisphosphate aldolase A	4		39 / 8.4	P05065
		Carbonic anhydrase II	5		29 / 6.88	P27139

‡ All identification were from the 2D gels in Figure 9 using LC MS/MS

* Also observed as nitrotyrosine immunoreactive following light exposure (Miyagi et al 2002)

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Conclusions

- Lipid oxidation products and oxidative protein modifications significantly increase in rat retina with intense light exposure, suggesting that they may serve as mediators in the mechanism of retinal light damage.
- Increased immunoreactivity of select photoreceptor proteins after light damage suggests that they may be more extractable, perhaps due to oxidative modifications and/or disruption of the photoreceptor outer segment membranes.
- Several rat retinal proteins possibly containing carboxyethyl pyrrole and/or argpyrimidine modifications were identified after light damage. Some of these proteins also appear to be nitrated following light exposure (Miyagi et al., 2002).
- AMD drusen and light damaged rat retina both appear to contain increased amounts of crystallin and carboxyethyl pyrrole adducts. We hypothesize that AMD retina also contains increased amounts of oxidative protein modifications. The rat light damage model may prove useful for testing the efficacy of various antioxidants and scavengers in reducing oxidative protein damage in retina.