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Lipid-derived and Other Oxidative Modifications of Retinal Proteins in a Rat Model of Smith-Lemli-Opitz Syndrome

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Abstract

Oxidative modification of proteins can perturb their structure and function, often compromising cellular viability. Such modifications include lipid-derived adducts (*e.g.*, 4-hydroxynonenal (HNE) and carboxyethylpyrrole (CEP)) as well as nitrotyrosine (NTyr). We compared the retinal proteome and levels of such modifications in the AY9944-treated rat model of Smith-Lemli-Opitz syndrome (SLOS), in comparison to age-matched controls. Retinas harvested at 3 months of age were either subjected to proteomic analysis or to immuno-slot blot analysis, the latter probing blots with antibodies raised against HNE, CEP, and NTyr, followed by quantitative densitometry. HNE modification of retinal proteins was markedly (>9-fold) higher in AY9944-treated rats compared to controls, whereas CEP modification was only modestly (2-fold) greater, and NTyr modification was minimal and exhibited no difference as a function of AY9944 treatment. Anti-HNE immunoreactivity was greatest in the plexiform and ganglion cell layers, but also present in the RPE, choroid, and photoreceptor outer segment layer in AY9944-treated rats; control retinas showed minimal HNE labeling. 1D-PAGE/Western blot analysis of rod outer segment (ROS) membranes revealed HNE modification of both opsin and β -transducin. Proteomic analysis revealed the differential expression of several retinal proteins as a consequence of AY9944 treatment. Upregulated proteins included those involved in chaperone/protein folding, oxidative and cellular stress responses, transcriptional regulation, and energy production. β A3/A1 Crystallin, which has a role in regulation of lysosomal acidification, was down-regulated. Hence, oxidative modification of retinal proteins occurs in the SLOS rat model, in addition to the previously

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described oxidation of lipids. The results are discussed in the context of the histological and physiological changes that occur in the retina in the SLOS rat model.

Keywords

Retina; Smith-Lemli-Opitz; protein modification; oxidation; oxidative stress; cholesterol

1. Introduction

The Smith-Lemli-Opitz syndrome (SLOS; OMIM #602858) is a rare, and often lethal, autosomal recessive human disorder caused by defective cholesterol biosynthesis (DeBarber et al., 2011; Nowaczyk and Irons, 2012). Mutations in the *DHCR7* gene, which encodes the enzyme 7-dehydrocholesterol reductase (EC 1.3.1.21) that catalyzes the last step in *de novo* cholesterol biosynthesis, lead to formation of a catalytically inefficient enzyme, resulting in physiologically abnormal and excessive accumulation of 7-dehydrocholesterol (7DHC) and reduced cholesterol levels in bodily tissues and fluids of affected individuals, relative to normal subjects. A viable rat model of SLOS has been achieved by treating normal rats with AY9944 (Fliesler et al., 2004), a relatively selective pharmacological inhibitor of DHCR7 (Dvornik et al., 1963; Kraml et al., 1964), which recapitulates the biochemical and some of the other phenotypic features of the human disorder. While the initiating event in the human disease is the genetic compromise of DHCR7 function, the sequelae are complex and not yet fully understood. Increasingly, multiple lines of the evidence suggest that oxidative stress plays an important and possible pivotal role in the pathobiology of SLOS, both in humans and in the rat model of SLOS (see *Discussion*). Key among these lines of evidence is the fact that 7DHC is extraordinarily susceptible to oxidation (Xu et al., 2009), forming a multitude of 7DHC-specific oxysterol byproducts, some of which are very toxic to cells (Korade et al., 2010; Xu et al., 2011, 2012; Pfeffer et al., 2016). Such 7DHC-derived oxysterols have been identified in tissue and serum specimens obtained from SLOS patients (Griffiths et al., 2017), as well as in tissues and serum obtained from the SLOS rat model (Xu et al., 2011, 2012), including the retina, but have not been found in appreciable amounts in tissues and serum from normal human or animal controls. In addition, a progressive and irreversible retinal degeneration has been described in the SLOS rat model (Fliesler et al., 2004); that degeneration can be completely prevented by dietary supplementation with cholesterol plus antioxidants (vitamins E and C plus selenium nitrite), but only partially by cholesterol supplementation alone, with concomitant reduction in the levels of 7DHC-derived oxysterols in tissues and serum (Fliesler et al., 2018). In addition, retinas isolated from the SLOS rat model exhibit markedly elevated levels of lipid hydroperoxides compared to age-matched controls (Richards et al., 2006). The levels of hydroperoxides can be further elevated (by >three-fold) by exposing the rats to intense constant light, but also can be down-regulated by pre-treating rats with a systemic antioxidant, such as dimethylthiourea (Vaughan et al., 2006). The severity of the associated retinal degeneration also correlates with the levels of lipid hydroperoxides found in the retina (Richards et al., 2006).

Heretofore, the main focus on the biochemical defect in SLOS has been on the cholesterol biosynthetic pathway. Relatively little attention has been paid to possible proteomic defects

associated with the human disease or its animal models. Tu et al. (2013) performed a detailed ion-current-based proteomic analysis of retinas from AY9944-treated and age- and sex-matched control Sprague-Dawley rats. They found 101 proteins had altered content, out of 1,259 proteins identified, including several associated with oxidative stress. However, that study was not designed to evaluate the presence of post-translational protein modifications, such as 4-hydroxy-2-nonenal (HNE), carboxyethylpyrrole (CEP), or nitrosylation of tyrosine residues (NTyr), which are also known to be associated with oxidative stress (Hardas et al., 2013), aging (Chakravarti, 2017), and retinal degenerations (Gu et al., 2003; Krilis et al., 2017). Here, we describe an initial study to examine such post-translational modifications, comparing retinas from AY9944-treated vs. age-/sex-matched control rats at a specific end point (3 postnatal months). The results obtained are discussed in the context of the progressive and irreversible retinal degeneration that is observed in the SLOS rat model, and their implications to understanding the pathobiology and treatment of human patients afflicted by SLOS.

2. Materials and methods

2.1. Materials.

Unless otherwise stated, biochemical and analytical reagents were used as purchased from Sigma/Aldrich (St. Louis, MO, USA). AY9944 (N-[(2-chlorophenyl)methyl]-1-[4-[(2-chlorophenyl)methylamino]methyl]-cyclohexyl]methanamine dihydrochloride) was custom synthesized by the Vanderbilt University Chemistry Core (Vanderbilt University, Nashville, TN, USA), and the structure and purity confirmed by ¹H-NMR and GLC-MS, in comparison with an authentic standard of the compound (a generous gift of Wyeth-Ayerst Laboratories, Radnor, PA, USA). 4-Hydroxy-2-nonenal (HNE) was used as purchased from Cayman Chemical (Ann Arbor, MI, USA). A rabbit polyclonal antibody reactive against HNE-modified proteins was purchased from Alpha Diagnostics (San Antonio, TX, USA). HNE-modified bovine serum albumin (HNEBSA) was prepared as previously described (Kapphahn et al., 2006). A mouse monoclonal antibody reactive against CEP protein adducts and a CEP-modified BSA standard were generous gifts of Dr. John Crabb (Cole Eye Institute, Cleveland Clinic Foundation; Cleveland, OH, USA). A mouse monoclonal antibody reactive against NTyr was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal antibodies against opsin/rhodopsin (RHO) and β -transducin (Gnb1) were obtained from Thermo Fisher Scientific (Grand Island, NY, USA). Antibodies were validated as described by Louie et al. (2002). Alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse), Kaleidoscope™ pre-stained molecular weight markers, Alkaline Phosphatase (AP) Conjugate Substrate Kit containing 5-bromo-4-chloro-3'-indoly-phosphate *p*-toluidine and nitro-blue tetrazolium chloride (BCIP-NBT), Silver Stain Plus™ Kit, and all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were supplied by Bio-Rad Laboratories (Hercules, CA, USA). AlexaFluor® 488-conjugated goat anti-rabbit IgG and DAPI (4',6-diamido-2-phenylindole) were purchased from Thermo Fisher Scientific. Fluoro-Gel™ and other materials and supplies utilized for paraffin-embedment microscopy/immunohistochemistry were purchased from Electron Microscopy Science (EMS, Hatfield, PA, USA).

2.2. Animals.

All procedures conformed to the National Research Council's *Guide for the Care and Use of Laboratory Animals* (<https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf>) and the *AVMA Guidelines for the Euthanasia of Animals* (2013 Edition; <https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>). The protocol involving the use of animals was reviewed and approved by the Institutional Animal Care and Use Committees (IACUCs) of Saint Louis University, the Veterans Administration Western New York Healthcare System, and the University at Buffalo. Rats were maintained in dim cyclic light (20–40 lux, 12 h light:12 h dark light cycle) at 22–25°C. The animal model of SLOS employed here was generated by treating rats with AY9944, as previously described (Fliesler et al., 2004). In brief, pregnant adult female Sprague Dawley rats (Harlan Bioproducts for Science, Indianapolis, IN, USA; 6 days post-fertilization) were implanted with an Alzet® osmotic pump (Model 2ML4; Durect Corp., Cupertino, CA, USA) containing a concentrated solution of AY9944 (in 0.01 MPBS, pH 7.4). They were fed a standard laboratory rat diet (Teklad 2016 16% Protein Global Rodent Diet®; Envigo, Madison, WI, USA), which is a plant-based, cholesterol-free chow. Pups were injected (*s.c.*) three times per week with AY9944 (in 0.01M PBS, pH 7.4; 30 mg/kg), starting on postnatal day 1 (PN 1) and continuing throughout the experimental time course (up to PN 90). In parallel, rats that were not treated at any time with AY9944 were maintained as age-matched controls, on the same standard rat chow.

2.3. Harvesting of retinas, preparation of retinal homogenates and ROS membranes.

At the end point of the experiment (3 postnatal months of age), rats were euthanatized by carbon dioxide inhalation, in compliance with the *American Veterinarian Medical Association (AVMA) Guidelines for Euthanasia* (2013 Edn.). Neural retinas (free of RPE and choroid) were harvested and flash-frozen in liquid nitrogen in screw-cap polypropylene microfuge tubes (Sarstadt; Newton, MA, USA), then stored at –80°C in darkness until ready for analysis. Flash-frozen retinas were homogenized in a glass Dounce homogenizer in a solution containing 250 µL 8M urea and 2% (w/v) CHAPS. Detergent-soluble and -insoluble fractions were separated by centrifugation at $16,000 \times g$ for 30 min using a tabletop microfuge, and the supernatant was kept as the soluble fraction. Protein concentrations were determined using BCA protein assay reagents (Pierce), with bovine serum albumin (BSA) as the standard protein. In a separate cohort, retinas were used to prepare isolated rod outer segment (ROS) membranes, using small-scale discontinuous sucrose gradient density ultracentrifugation, as described in detail previously (Fliesler et al., 1995; Fliesler et al., 2000).

2.4. Proteomic analysis of retinal proteins.

The methods employed for separation of proteins by one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) and Western blot analysis were as described in detail previously (Louie et al., 2002; Kapphahn et al., 2006). Protein loads were 15 µg per lane for 1D gels and 90 µg for 2D gels. After electrophoresis, gels were stained silver with a mass spectrometry-compatible kit (Silver Stain Plus™ Kit; Bio-Rad) and imaged with a fluorescence imager (Fluor-S Multi-Imager; Bio-Rad). Spot intensities (spot volume) were

quantified using PDQuest™ v.7.1.1 software (Bio-Rad). This program performs gel-to-gel comparisons by using algorithms that account for differences in background and staining intensities. Gel-to-gel spot matching was performed by generating a master gel and manually selecting 12 spots that were consistently present in all gels as landmarks. Automatic spot detection was followed by manual inspection and spot editing to ensure consistent matches between gels. Statistical analyses were performed with log2-transformed spot intensities.

Peptide extraction from polyacrylamide gels, peptide mass fingerprinting, and sequencing by tandem MS/MS were performed as described (Nordgaard et al., 2006) with the following modifications. Data were collected on an Orbitrap LC-MS (Thermo Fisher Scientific), or a 4800 TOF/TOF (Sciex, Framingham, MA, USA). Peptide masses were analyzed using ProteinPilot™ v.4.5 software (Sciex, Framingham, MA). The peptide mass fingerprinting searches used mass tolerance settings of 50 ppm, carbamidomethylation of cysteine and oxidation of methionine (processing artifacts) as fixed and variable modifications, respectively, and missed cleavages by trypsin. Protein identifications were considered verified by Western blotting or by sequencing if the MS/MS spectra were at 99% confidence level and by manual inspection of spectra.

2.5. Slot-blot analysis of oxidative modifications.

Slot blot analysis of total retinal homogenates was performed as previously described (Ferrington et al., 2002; Kapphahn et al., 2006). Retinal homogenates and standards of BSA modified with HNE, CEP, or NTyr were adsorbed to polyvinylidene difluoride (PVDF) membranes using a slot blot apparatus (Bio-Dot® SF, Bio-Rad). Primary antibodies (1:1000 dilution) were used in conjunction with species-appropriate alkaline phosphatase-conjugated secondary antibodies (1:3000 dilution) and the chromogenic substrate BCIP-NBT to visualize the immunospecific reactions, which were quantified by densitometric analysis of scanned slot blots using SigmaScan® software (Systat Software, Inc., San Jose, CA, USA).

2.6. Immunohistochemistry.

Freshly enucleated eyes were immersed in PBS-buffered 3.7% formaldehyde (prepared from paraformaldehyde), on ice for 15–30 minutes, and then rinsed in PBS three times. Fixed tissue was then processed for paraffin embedment and sectioning as described in detail previously (Fliesler et al., 2004), under a fee-for-service agreement with the Research Microscopy and Histology Core, Department of Pathology, Saint Louis University (St. Louis, MO, USA). Immunohistochemistry was performed essentially as described in detail previously (Fliesler et al., 2018). In brief, tissue sections (10-µm thickness) from paraffin-embedded eyes were collected onto Superfrost™Plus glass microscope slides. Following deparaffinization/rehydration and antigen retrieval, sections were first treated with nonimmune (normal) goat serum (5% (v/v) in TBST (Tris-buffered saline supplemented with 0.2% (v/v) Tween-20) containing 0.5% (w/v) BSA and 0.5% (w/v) fish skin gelatin), in order to block non-specific binding of secondary antibodies (host origin: goat). This was followed by incubation (1 h, at room temperature) with in primary antibody (rabbit anti-HNE; 1:500, diluted in TBST supplemented with 0.5% (w/v) BSA). After three rinses with TBST, tissue sections were incubated for 45 min at room temperature with AlexaFluor®

488-conjugated goat anti-rabbit IgG (1:500 dilution, as above for primary antibody). Slides were rinsed with TBS, counterstained by applying DAPI (working concentration, 1 µg/ml), then rinsed in distilled water, mounted on glass microscope slides using Fluoro-Gel™, coverslipped, and examined with a Leica TCS SPEII DMI4000 scanning laser confocal fluorescence microscope. Images were captured using the 40× oil immersion (RI-1.518) objective under nominal laser intensity (10%–20% of maximum intensity), arbitrary gain (800) and offset (–0.2) values, to optimize the signal-to-noise ratio.

2.7. Statistical analysis.

Quantitative data were statistically analyzed using a two-tailed Student's *t*-test; a value of *p* < 0.05 was set as the threshold for statistical significance.

3. Results

3.1. Retinal proteins are oxidatively modified in the SLOS rat model.

Figure 1 shows results from a slot blot analysis of total neural retina proteins obtained from retinas (N=3 each) from AY9944-treated and age-matched control rats, at three months of age. All three antibodies tested (anti-HNE, -CEP, and -NTyr) exhibited immunoreactivity to retinal proteins, notably even in the control samples. AY9944 treatment caused a nearly nine-fold (*p* < 0.05) increase in the intensity of the HNE-immunoreaction of retinal homogenates, compared to age-matched control samples. The difference between AY9944-treated vs. control immunoreactivity to anti-CEP was less pronounced, but was still nearly two-fold and statistically significant. However, there was no statistically significant difference observed in anti-NTyr immunoreactivity as a function of AY9944 treatment, relative to the control specimen.

3.2. Immunolocalization of HNE-modified proteins in rat retina.

Since slot blot analysis revealed only a modest difference in immunolabeling of retinal proteins using anti-CEP and no difference for the anti-NTyr antibodies, we did not pursue further analyses with these two antibodies. Rather, we examined the distribution of anti-HNE immunoreactivity as a function of retinal cell layer and AY9944 treatment by immunofluorescence histochemistry. Results are shown in Figure 2 for representative retinas from an AY9944-treated vs. an age-matched control rat (three months postnatal age). The control retina exhibited only minimal (background) immunostaining, except in the choroid (Chor). By contrast, the retina from an AY9944-treated rat exhibited intense immunostaining, particularly in the inner and outer plexiform layers (INL, OPL) and ganglion cell layer (GCL), as well as in the remnant photoreceptor outer segment (OS) layer, retinal pigment epithelium (RPE), and choroid (Chor). Note the marked reduction in total retinal thickness of the AY9944-treated retina, relative to the age-matched control retina, consistent with the previously demonstrated advanced stage of retinal degeneration at this time point (Fliesler et al., 2004).

3.3. Opsin and β-transducin are HNE-modified in rod outer segments.

The results shown in Fig. 2 indicate that anti-HNE immunoreactivity was increased in the photoreceptor outer segment (OS) layer by AY9944 treatment, although more modestly than

it was in the INL, OPL, GCL, and RPE layers. Since our prior studies have demonstrated that retinal degeneration and cell death in the SLOS rat model is almost exclusively restricted to the photoreceptor layer (Fliesler et al., 2004; Tu et al., 2013), we pursued biochemical analysis of rod OS (ROS) membranes isolated from AY9944-treated and control rats (three months postnatal age).

ROS membranes were subjected to 1D-SDS-PAGE and Western blot analysis, probing the blot with anti-HNE and anti-opsin antibodies (Fig. 3). The silver-stained PAGE gel (Fig. 3A) shows that opsin (monomer (*), $M_r \sim 36$ kDa) was, by far, the dominant protein in ROS membranes, in good agreement with numerous prior reports. There were no qualitative differences in the staining pattern of the gel as a function of AY9944 treatment, and the protein loads appeared equal. In order to reveal the presence of other ROS-specific proteins of comparable M_r to opsin, but which might be masked by opsin, we compared the staining patterns with (+) and without (–) previously boiling the ROS samples in Laemmli sample buffer. This treatment denatures proteins, but also promotes oligomerization of opsin and other multi-helical membrane proteins (Dutta et al., 2010; Sagné et al., 1996; Taylor et al., 2000). Of note, the silver staining patterns in boiled samples were comparable in the AY9944-treated and control specimens. As expected, boiling caused a marked decrease in the silver staining intensity of the band corresponding to opsin monomer, and a substantial increase in staining of higher molecular weight bands, particularly those migrating with $M_r > 97$ kDa, consistent with opsin oligomers.

Blots were probed with antibodies that recognize either opsin (Fig. 3B) or HNE-modified proteins (Fig. 3C). To validate the anti-HNE antibody, we used HNE-modified and unmodified BSA as positive and negative controls, respectively. As shown in Fig. 3C (far right-hand panel, lanes labeled M and U), only the modified BSA exhibited a positive immune reaction, confirming the specificity of this antibody. The Western blot of ROS proteins showed a multiplicity of HNE-positive bands, but with the dominant band having a M_r comparable to that of opsin monomer, both in AY9944-treated and control specimens. Notably, after boiling, the intensity of that HNE-positive band markedly *increased*, both in the AY9944-treated and control specimens; however, there was no increase in HNE-immunopositive bands at higher M_r values, particularly at positions corresponding to opsin oligomers. These findings suggest that while monomeric opsin can be modified by HNE under the experimental conditions employed, opsin oligomers either are relatively resistant to HNE modification, or the HNE-derivatized epitopes (primarily lysine residues) are inaccessible to antibody binding. Alternatively, the efficiency of electrophoretic transfer of high-molecular weight oligomers of opsin may be low, compared to that of opsin monomer and other medium to small M_r proteins, hence giving the misleading impression that they are not reactive to anti-HNE.

Since other proteins migrating at $M_r \sim 36$ kDa also might contribute to the anti-HNE immune reaction, we performed MALDI-TOF analysis of this band with a QSTAR XL mass spectrometer (Sciex, Framingham, MA). Mass spectrometry identified β -transducin subunit 1 (control sample: 12 peptides/ 41% coverage; AY sample: 9 peptides/ 24% coverage) within this region of the gel. To determine if β -transducin is HNE-modified, we performed 2D-IEF/PAGE followed by Western blot analysis, probing with antibodies against β -transducin and

then reprobed the blots with anti-HNE. Our rationale for this approach was that since membrane proteins do not resolve on 2D gels, opsin would not appear on the blots, thus allowing us to identify other HNE-modified proteins of similar M_r . The results (Fig. 3D, *upper panels*) show that the reaction of β -transducin-specific antibody is positioned at M_r ~36 kDa, but runs as a smear across a pH range from 3 to 6 (theoretical $pI = 5.47$) in both the untreated control and AY9944-treated samples. Migration of a protein through a range of isoelectric points results from multiple post-translational modifications that alter the intrinsic charge of the protein (Dunn, 1987).

Reprobing these blots with the antibody to HNE (Fig. 3D, *lower panels*) showed a similar pattern of immunoreaction, and at the same M_r , indicating that in addition to monomeric opsin, β -transducin was also HNE-modified. Of note, these HNE-modified proteins were found to be present in ROS membranes from both untreated and AY9944-treated rats (see *Discussion*). Although the Western blots may give the initial impression that the levels of HNE-modified proteins were greater in ROS specimens from AY9944-treated rats, compared to untreated controls, we cannot make this claim definitively, because one cannot reliably compare densitometric values from one blot to another. That said, the immunohistochemistry results (Fig. 2) clearly indicate that there is much greater anti-HNE immunoreactivity in AY9944-treated retinas compared to controls, including in the ROS layer.

3.4. AY9944 treatment causes up-regulation of stress-related proteins.

In order to examine the identities of retinal proteins that were altered in their expression levels as a function of AY9944 treatment, we subjected retinal homogenates from AY9944-treated *vs.* age-matched control rats (three months postnatal age) to 2D-IEF/PAGE. Expression analysis then was conducted on silver-stained gels and the protein spots that exhibited significant changes in density with AY-treatment were identified by MALDI-TOF mass spectrometry. Fig. 4 and Table 1 show the results obtained. The relative levels of 16 proteins were up-regulated and five proteins were down-regulated by AY9944 treatment, compared to controls. Mass spectrometry was able to identify 62% of these proteins with altered expression. Table 1 summarizes the protein identifications from 12 up-regulated and one down-regulated protein spots. The following were among those proteins that were up-regulated: peroxiredoxin 6, protein disulfide isomerase (PDI), pirin, vimentin, and glial fibrillary acidic protein (GFAP). Several proteins also were found to be down-regulated by AY9944-treatment; however, among these down-regulated proteins, we were only able to conclusively identify $\beta A3/A1$ crystallin. Overall, spot migration position was in good agreement with the theoretical mass of the identified proteins (Table 1). For three protein spots (#4, #5, and #10), multiple proteins were identified. Based on the difference between the spot migration (# 5, ~50 kDa; # 10, ~ 24 kDa) and the theoretical mass of glutamate oxaloacetate transaminase (92 kDa) and GFAP (50 kDa), we can conclude that the peptides that were identified in spots #5 and #10 were likely degradation products of the native, intact protein originally present in the specimens.

Western blotting was used as a secondary analysis on select proteins to validate the identities of protein components revealed by 2D-IEF/PAGE and MALDI-TOF analysis (Fig. 4C).

Multiple crystallin proteins were identified by our 2D gel analysis, including α A, α B, and β A3/A1 crystallins. Both α A and α B crystallins undergo multiple post-translational modifications that can alter the isoelectric point, causing a change in migration across the pH gradient of the 2D gel (Colvis and Garland, 2002). Thus, the population of these proteins runs as a “charge train” (also observed for β -transducin), rather than as a discrete single spot. One additional complication for α A crystallin is that in rodent retina, a second protein isoform is produced from an alternate gene product that inserts 23 amino acids between residues 63 and 64 of the α A crystallin gene (King et al., 1984). The resultant protein is called “ α A insert” and is likely associated with spot #11. As we have previously shown, these two forms of α A can be resolved by 1D-PAGE/Western blot analysis (Kapphahn et al., 2003). Analysis of the total content of α A, α A insert, and α B crystallin showed they were unchanged with AY9944 treatment, indicating that only a portion of each protein’s total spot population (# 6, 9, 10, 11) was up-regulated in AY9944-treated rats. In contrast, analysis of total protein for β A3 crystallin was in good agreement with the results of 2D-IEF/PAGE analysis (spot #13). Both analyses showed that the levels of β A3 crystallin were barely detectable by the methods employed.

No appreciable differences in total protein levels were observed for glutamate oxaloacetate transaminase (#5) or transaldolase I (#4), proteins whose peptides were identified in spots found to have multiple protein constituents. Vimentin was found in two up-regulated spots (#4 and #5), but total protein levels were not significantly different. The levels of GFAP, found as the sole protein in spot #8 and in the multi-protein spot #10, were significantly ($p < 0.05$) up-regulated with AY9944 treatment, relative to controls.

Taken together, these results demonstrate that oxidative modification and alteration in expression levels of retinal proteins occur under conditions that have been shown to promote and exacerbate oxidation of lipids and retinal degeneration in the SLOS rat model (Fliesler et al., 2018; Richards et al., 2006; Vaughan et al., 2006; Xu et al., 2011, 2012).

4. Discussion

Herein, we’ve demonstrated that oxidative modification of retinal proteins occurs in the AY9944-induced rat model of SLOS. Since such modifications are known to compromise the structure and function of proteins (Berlett and Stadtman, 1997; Domingues et al., 2013; Yakubenko and Byzova, 2017), this must be taken into account in addition to the primary biochemical defect in cholesterol biosynthesis as well as the oxidation of lipids, including sterols, in understanding the pathophysiological mechanism underlying the associated retinal degeneration in this animal model (Fliesler, 2010; Fliesler, 2014). To the extent that this model is faithfully relevant to the human disease it is purported to model (SLOS), these findings have translational implications for developing suitable therapeutic strategies for intervening in SLOS, *i.e.*, by blocking or reducing the extent of such oxidative modifications. As proof of principle, we recently showed (Fliesler et al., 2018) that feeding AY9944-treated rats a high-cholesterol diet enriched in antioxidants completely prevented the retinal degeneration associated with this SLOS animal model, as well as lowering the levels of 7-dehydrocholesterol-derived oxysterols in the retina, whereas a high-cholesterol diet alone only partially protected the retina from degeneration and did not provide

statistically significant lowering of oxysterol levels. It would be of interest to revisit those experiments, assaying for the types and amounts of oxidatively modified proteins (*e.g.*, HNE-modified opsin and β -transducin), to assess whether the antioxidant regimen reduced the extent of those oxidative modifications.

Given that we observed HNE modification of proteins which is derived from oxidation of ω 6 polyunsaturated fatty acids, such as arachidonic acid (20:4, n-6), and had previously also observed a profound decrease in the DHA (22:6, n-3) content of the retina and ROS membranes in the SLOS rat model, compared to controls (Ford et al., 2008), we had originally anticipated that we might also observe more generalized oxidative modification of proteins with CEP (carboxyethylpyrrole), an aldehyde specifically derived from oxidative degradation of DHA (Gu et al., 2003; Renganathan et al., 2008), as well as HHE (4-hydroxy-2-hexenal), an aldehyde by product of ω 3 polyunsaturated fatty acid oxidation (Long and Picklo, 2010; Van Kuijk et al., 1990; Yamada et al., 2004). However, a separate set of experiments failed to consistently demonstrate evidence for such other modifications as a consequence of treatment of rats with AY9944 (data not shown).

The finding that the levels of GFAP and vimentin were significantly ($p < 0.05$) up-regulated with AY9944 treatment relative to controls is consistent with the fact that these proteins are intermediate filament proteins and biomarkers of glial cells and astrocytes (Lewis and Fisher, 2003; Hermann and Aebi, 2016). These results are also consistent with our previous reports that gliosis and marked up-regulation of GFAP occur in the course of the AY9944-induced retinal degeneration in this SLOS animal model (Fliesler et al., 2004; Tu et al., 2013). With regard to some other retinal proteins whose expression levels were found to be upregulated by AY9944 treatment, relative to age-matched controls, representative members from several protein families were identified. For example: peroxiredoxin 6 (Prdx6), which controls cellular peroxide levels and, hence, is important in the ability of cells to combat oxidative stress (Wood et al., 2003; Fisher, 2011); protein disulfide isomerase (PDI) A3, which is located in the endoplasmic reticulum (ER), catalyzes disulfide bond formation, and is important in normal protein folding and combatting ER stress (Soares Moretti and Martins Laurindo, 2017); transaldolase 1 (TALDO1), a component of the pentose phosphate pathway, which produces NADPH (reducing equivalents for cellular anabolic reactions) and pentose precursors for nucleotide biosynthesis (Tsolas and Horecker, 1973; Samland and Sprenger, 2009); triose phosphate isomerase, a key glycolytic enzyme central to cellular energy metabolism (Gracy, 1982; Wierenga et al., 2010); pirin, an Fe(II)-containing nuclear protein and transcriptional co-regulator of NF κ B genes involved in regulation of apoptosis (Pang et al., 2004; Liu et al., 2013); and chromobox protein homolog 3 (CBX3), another gene transcriptional regulator (Singh, 2010).

Conversely, we found that β A3/A1 crystallin was significantly down-regulated in the retina as a consequence of AY9944 treatment, relative to controls. This protein, originally identified in the ocular lens (Piatigorsky, 1989), has been shown to be present also in the developing postnatal rat retina, particularly astrocytes and ganglion cells (Parthasarathy et al., 2011) as well as in astrocytes and RPE cells in the mouse retina (Valapala et al., 2016). In the RPE, β A3/A1 crystallin is trafficked to lysosomes, where it regulates the V-ATPase complex responsible for generating the pH gradient that maintains the acidic intralumenal

microenvironment of the lysosome (Zigler and Sinha, 2015; Valapala et al., 2016). Conditional ablation of the *Cryba1* gene in the RPE results in altered lysosomal pH, reduced activity of lysosomal enzymes, and incomplete degradation of phagocytized ROS. Hence, significantly reduced levels of β A3/A1 crystallin in the retinas of AY9944-treated rats would be expected to impair normal lysosomal function, including the autophagic degradation and clearance of intracellular proteins (Larsen and Sulzer, 2002; Wang and Klionsky, 2003). However, defective autophagic flux does not necessarily involve impaired lysosomal function, as we've recently demonstrated using both the AY9944 SLOS rat model and iPSC-derived RPE cells from SLOS patients (Ramachandra Rao et al., 2018).

Collectively, these changes in protein expression levels make good biological sense when considered in the context of the global structural and physiological changes the retina undergoes in the course of the AY9944-induced retinal degeneration (Fliesler et al., 2004). As previously suggested (Fliesler, 2010; Fliesler, 2014), while the primary defect in SLOS as well as in the AY9944 SLOS rat model is disruption of cholesterol synthesis (and abnormal accumulation of its immediate precursor, 7DHC), the ensuing pathological sequelae involve a multitude of cellular processes and biochemical reactions that go well beyond this initial defect. Hence, this additional complexity in the pathobiological mechanism of SLOS must be taken into account when developing new therapeutic interventions for this disease. In the absence of a viable and reliable genetic animal model of SLOS, the AY9944 SLOS rat model offers a suitable preclinical biological system for achieving that goal.

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Abbreviations:

1D	one-dimensional
2D	two-dimensional
7DHC	7-dehydrocholesterol
BSA	bovine serum albumin

BTrans	β -transducin
CEP	carboxyethylpyrrole
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHOL	cholesterol
Chor	choroid
DAPI, 4'	6-diamido-2-phenylindole
DHCR7	7-dehydrocholesterol reductase
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
GLC	gas-liquid chromatography
HHE	4-hydroxy-2-hexenal
HNE	4-hydroxy-2-nonenal
IEF	isoelectric focusing
INL	inner nuclear layer
IPL	inner plexiform layer
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
M_r	apparent molecular weight
MS	mass spectrometry
NMR	nuclear magnetic resonance
NTyr	nitrotyrosine
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	photoreceptor outer segment layer
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulfide isomerase
pI	isoelectric focusing point
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
SLOS	Smith-Lemli-Opitz syndrome

TBS	Tris-buffered saline
TBST	TBS containing Tween-20

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Highlights

- Oxidative modification of retinal proteins, including opsin and β -transducin, occurs in the AY9944-induced rat model of SLOS.
- 4-Hydroxy-2-nonenal (HNE) protein adducts are significantly elevated in the AY9944-induced rat model of SLOS.
- Expression levels of multiple protein family members were altered by AY9944 treatment, particularly up-regulation of stress-related proteins.

Figure 1. Slot blot analysis of total retinal proteins from AY9944-treated (AY) vs. control rats.
A. Individual blots probed with monospecific antibodies to carboxyethylpyrrole (CEP), 4-hydroxy-2-nonenal (HNE), and nitrotyrosine (NTyr), followed by alkaline phosphataseconjugated secondary antibodies and chromogenic substrates. **B.** Densitometric quantification of slot blots. Values (mean ± SEM) are normalized to the average value for control samples. Asterisk (*) denotes statistically significant difference ($p < 0.05$, Student's t -test) between treated ($n=3$) and control ($n=3$) samples.

Figure 2. Immunofluorescence histochemical detection of anti-HNE immunoreactive proteins (green) in retinas from control (left panel) vs. AY9944-treated (right panel) rats.

Postnatal 3 months of age. Note minimal label intensity of all retinal layers in the control specimen, except for the choroid (Chor), compared to the markedly intense immunolabeling of several retinal layers in the AY9944-treated specimen, particularly the plexiform layers, outer segment (OS) layer, RPE, and Chor. Note also the marked thinning of the AY9944-treated retina, compared to the age-matched control retina, consistent with the known retinal degeneration associated with this SLOS animal model. DAPI counterstain (blue, far left panel) highlights location of nuclei in the retinal cell layers. *Abbreviations:* Chor, choroid; OS, outer segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar (all panels), 40 μ m.

Figure 3. SDS-PAGE and companion Western blot analysis of ROS proteins from AY9944-treated (AY) and control (Con) rats.

Samples were either boiled (+) or not (-) in sample buffer prior to loading onto PAGE gel.

A. 1D-SDS-PAGE (*silver stain*) of ROS proteins, with migration position of opsin monomer denoted by asterisk (*). Note marked reduction in opsin monomer staining density and appearance of densely-stained high molecular weight bands (>97 kDa) upon boiling, consistent with opsin oligomerization. **(B, C)** 1D Western blots, probed with either anti-opsin **(B)** or anti-HNE **(C)** antibodies. Note marked enhancement of HNE-positive band, running at M_r similar to that of opsin monomer upon boiling, compared to the sample that was not boiled. **(C, far right-hand panel)** shows Western blot of HNE-modified (M) and unmodified (U) BSA standard (positive and negative antibody controls, respectively). **D.** 2D-IEF/PAGE Western blots, probed with antibodies either to β -transducin (BTrans; *upper panels*) or HNE (*lower panels*). Note exact coincidence of BTrans and HNE immunostaining. Apparent molecular weight (M_r , in kDa) of protein standards indicated by numbers in left-hand side of panels.

Figure 4. Proteomic analysis (2D-IEF/PAGE and mass spectrometry) of retinal proteins from AY9944-treated (AY) and control rats.

A. Silver-stained 2D gel showing the proteins that had altered expression levels (*boxes*), AY9944-treated *vs.* control (*red* box, up-regulated; *blue* box, down-regulated). Proteins within circled spots were not identified. **B.** Individual stained protein spot densities were normalized to the average spot density for control samples. All proteins were significantly different ($*p < 0.05$; two-tailed Student's *t*-test) after comparing spot density of specimens from control ($n=9$) *vs.* AY9944-treated ($n=5$) rats. Protein identifications are provided in Table 1. **C.** Western blot analysis of proteins identified in the proteomic analysis with altered expression. $*p=0.01$, determined by Student's *t*-test comparing AY-treated ($n=9$) and control ($n=8$) samples. Abbreviations: α A, α A crystallin; α A I, α A crystallin insert; α B, α B crystallin; β A3, β A3 crystallin; GOT1, glutamate oxaloacetate transaminase 1; Tra, transaldolase 1; GFAP, glial fibrillary acidic protein; Vim, vimentin

Table 1.

Protein Identification by Mass Spectrometry

Spot#	Protein	UniProt Accession #	Theoretical Mass (kDa)	Agreement w/spot migration	P Value	# peptides MS/MS	MALDI-TOF % Coverage	Western Blot
1 ^a	Protein Disulfide Isomerase A3	P11598	58	×	0.03	2	5	
2 ^b	Peroxiredoxin 6	Q35244	25	×	0.039	2	10	
3 ^a	Pirin	Q5M827	32	×	0.006	2	6	
4 ^a	Vimentin	P31000	54	×	0.037	4	8	×
	Transaldolase I	Q9EQS0	38	×	0.037	4	11	×
5 ^a	Vimentin	P31000	54	×	0.03	2	4	×
	Glutamate oxaloacetate transaminase I	P13221	92		0.03	4	9	×
6 ^b	αB Crystallin	P23928	22	×	0.013	6	43	×
7 ^b	Triose Phosphate Isomerase I	P48500	27	×	0.035	9	48	
8 ^b	Glial Fibrillary Acidic Protein	P47819	50	×	0.009	5	11	×
9 ^b	αA Crystallin	A0JN13	22/19	×	0.004	8	47	×
10 ^a	Glial Fibrillary Acidic Protein	P47819	50		0.05	5	11	×
	Chromobox Homolog 3	Q5RJK5	22	×	0.05	2	13	
	αB Crystallin	P23928	22/19	×	0.05	2	11	×
11 ^b	αA Crystallin	A0JN13	22/19	×	0.05	4	35	×
12 ^b	Triose Phosphate Isomerase I	P48500	27	×	0.05	6	25	
13 ^a	βA3 Crystallin	P141881	25	×	n/a*	2	12	×

^a data collected on Orbitrap;^b data collected on 4800 TOF/TOF;

* absent in AY samples