Evidence That Light Modulates Protein Nitration in Rat Retina*

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As part of ongoing efforts to better understand the role of protein oxidative modifications in retinal pathology, protein nitration in retina has been compared between rats exposed to damaging light or maintained in the dark. In the course of the research, Western methodology for detecting nitrotyrosine-containing proteins has been improved by incorporating chemical reduction of nitrotyrosine to aminotyrosine, allowing specific and nonspecific nitrotyrosine immunoreactivity to be distinguished. A liquid chromatography MS/MS detection strategy was used that selects all possible nitrotyrosine peptides for MS/MS based on knowing the protein identity. Quantitative liquid chromatography MS/MS analyses with tetranitromethane-modified albumin demonstrated the approach capable of identifying sites of tyrosine nitration with detection limits of 4–33 fmol. Using two-dimensional gel electrophoresis, Western detection, and mass spectrometric analyses, several different nitrotyrosine-immunoreactive proteins were identified in light-exposed rat retina compared with those maintained in the dark. Immunocytochemical analyses of retina revealed that rats reared in darkness exhibited more nitrotyrosine immunoreactivity in the photoreceptor outer segments. After intense light exposure, immunoreactivity decreased in the outer segments and increased in the photoreceptor inner segments and retinal pigment epithelium. These results suggest that light modulates retinal protein nitration in vivo and that nitration may participate in the biochemical sequelae leading to light-induced photoreceptor cell death. Furthermore, the identification of nitrotyrosine-containing proteins from rats maintained in the dark, under nonpathological conditions, provides the first evidence of a possible role for protein nitration in normal retinal physiology.

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Nitrination of tyrosine is one of several protein modifications that can occur as a result of oxidative stress (1). A number of inflammatory and neurodegenerative disorders have been associated with tyrosine nitrination including ocular inflammation, retinal ischemia, lung infection, cancer, Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease (2). The abundance of this protein modification is low, perhaps less than 1 in $10^5$ tyrosine (3), and identification of all in vivo protein nitration targets have utilized antibodies directed against nitrotyrosine (4). The only successful identification of in vivo sites of tyrosine nitration to date has been achieved with histone H2B from mouse tumors, a relatively abundant protein that may accumulate more nitrotyrosine than the average cellular protein because of greater stability and lower turnover (5). Previously we used a combination of immunological detection and MALDI-TOF MS to identify proteins nitrated on tyrosine during inflammatory challenge (4) and now focus on a possible link between protein nitration and retinal light damage. Partial protection from retinal light damage has been observed in rats treated with N-nitroarginine methyl ester (6), an inhibitor of nitric-oxide synthase. Because nitric-oxide synthase provides the in vivo precursor for nitrating agents, nitration may play a role in light-induced retinal degeneration.

Retinal damage from intense visible light was reported first in 1966 (7) yet the molecular mechanisms remain poorly understood. Light-induced photoreceptor cell degeneration appears to involve both apoptosis and necrosis (8–10) via a process initiated by oxidative stress, because antioxidants such as ascorbate and dimethylthiourea provide protection (8, 10). Damaging light is thought to produce reactive oxygen species; however the identity of such species and the pathways of oxidative damage remain unknown (10). The similarity in the action spectrum of retinal light damage and the absorption spectrum of rhodopsin (~500 nm maximum) has lead to the hypothesis that injury may be initiated by rhodopsin bleaching (7, 11). Additional evidence supports the involvement of retinoids in the process. Blue light induces apoptosis

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* The abbreviations used are: MALDI-TOF, matrix assisted laser desorption ionization time of flight; BSA, bovine serum albumin; IEF, isoelectric focusing; LC, liquid chromatography; MS/MS, tandem mass spectrometry; NO, nitric oxide; N,Y, nitrotyrosine; PBS, phosphate-buffered saline; PVDF: polyvinylidine difluoride; RPE, retinal pigment epithelium; ROS, rod outer segments; 2D, two-dimensional; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
in cultured retinal pigment epithelial cells in a manner related directly to the content of pyridinium bisretinoid, i.e. lipofuscin component A2E (12). In vitro, light induces aggregation of the photoreceptor-specific protein ABCR in the presence of all-trans-retinal (13). Dietary vitamin A-depleted rats (14), as well as animals with impaired visual cycle retinoid processing proteins such as CRALBP (15), RPE65 (16), and RDH5 (17), appear to be more resistant to light damage than normal control animals. However age, diet, genetics, and environmental light history all influence the extent and type of retinal injury from light exposure (8).

The purpose of this study was to improve methods for characterizing protein nitration and to investigate whether protein nitration is a mediator in photoreceptor light damage. An improved 2D gel Western method for detecting nitrated proteins is described, and a quantitative baseline is established for LC MS/MS determination of sites of tyrosine nitration. We have also surveyed the nitroproteome of retina from rats exposed to intense light or maintained in the dark. The results indicate that the distribution and identity of nitroproteins are similar within the cell layers of the retina when exposed to light.

**Experimental Procedures**

**Animal Procedures and Retinal Preparations**—Albino male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN) were received at 21 days of age and maintained in darkness until use. The dark environment was interrupted with dim red illumination about 30 min per day during routine animal care. The rats were fed a standard rat chow (Teklad, Madison, WI) and given water ad libitum. Rats maintained in a dark environment for 2–4 months were exposed to intense (1500 lux) green light (490–580 nm) for 3 h and sacrificed immediately following light treatment under dim red illumination in a chamber with a CO₂-saturated atmosphere. All procedures involving rats followed the protocols outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four animals were used for 2D gel Western analyses of retinal proteins, and eight animals were used for immunocytochemistry. Retinas were excised from rats within 2 min of death as described by Delmelle et al. (18), rinsed in PBS, and frozen in liquid nitrogen. Photoreceptor rod outer segments (ROS) were isolated from retinas using a discontinuous sucrose gradient technique as described by Organisciak et al. and stored at −80 °C until use.

**Gel Electrophoresis**—Each fresh retina was homogenized in 50 μl of isoelectric focusing (IEF) solvent B (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 2% carrier ampholine, pH 3–10, 1% dithiothreitol) using a disposable pestle at 4 °C. The homogenate was centrifuged at 14,000 rpm/min (Eppendorf table tap centrifuge 5415C) for 30 min, and the supernatant was collected. Photoreceptor ROS were incubated in PBS containing 1% dodecyl-β-maltoside at 4 °C for 1 h. Sucrose in the ROS solution was removed by repeated concentration and dilution with the same buffer using an ultracentrifuge filter tube (5-kDa molecular mass cut-off; Millipore, Bedford, MA). Lipids were extracted from ROS with an equal volume of chloroform:methanol (2:1 v/v), and proteins in the aqueous phase were collected, dried, and redissolved in IEF solvent B. Proteins in retinal and ROS extracts were quantified by a modified Bradford procedure (20). For one-dimensional gel electrophoresis, retinal extracts and ROS preparations in solvent B were diluted 1:1 with Laemmli SDS-PAGE sample buffer, and electrophoresis was performed according to Laemmli (21) using the Bio-Rad Mini-Protein II system.

Two-dimensional gel electrophoresis was performed with the IPGphor/IsoDalt systems (Amersham Biosciences) as described by West et al. (22). First dimension IEF was performed with the IPGphor system in IEF solvent B using 18 cm of non-linear pH 3–10 immobilized pH gradient strips and a programmed voltage gradient (22). After the first dimension IEF, the strips were incubated in 10 ml of reducing solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 100 mM dithiothreitol, 30% glycerol, 2% SDS) for 15 min at room temperature and then in 10 ml of alkylation solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 250 mM iodoacetamide, 30% glycerol, 2% SDS) for 15 min at room temperature. For the second dimension, the immobilized pH gradient strips were embedded in 0.7% (w/v) agarose on the top of 12% acrylamide slab gels (23.5 × 18.0 × 0.15 cm) containing a 4% stacking gel, and SDS-PAGE was performed overnight at 20–30 mA/gel with the IsoDalt system (22). Gels were stained with colloidal Coomassie Blue (Pierce Code Blue) according to the vendor. Detection limits of the stain were determined to be −15 ng of protein on 1.5-mm-thick gels based on quantitative analyses with BSA (quantified by amino acid analysis) and a commercial preparation of molecular weight markers (catalogue number 161–0304; Bio-Rad).

**Western Analysis**—For Western blot analysis, proteins in 2D gels were partially transferred to PVDF membranes (Millipore, Bedford, MA) at 320 mA/gel for 25 min using a Semi Dry Transfer Cell (Bio-Rad). Proteins remaining in the gels were visualized by colloidal Coomassie Blue and excised for identification. Two PVDF membranes were used for each set of Western analysis. One membrane was chemically reduced before Western analysis, and another membrane was used for Western analysis without reduction. Reduction of nitrotyrosine to aminotyrosine was achieved by treating one membrane with 10 mM sodium dithionite in 50 mM pyridine-acetate buffer, pH 5.0, for 1 h at room temperature. After the reaction, the membrane was rinsed with distilled water and then equilibrated with wash solution 1 (20 mM Tris, 150 mM NaCl, pH 7.5, 0.2% Tween 20). The reduced and non-reduced PVDF membranes were blocked for 1 h at room temperature in blocking solution (20 μl Tris, 150 mM NaCl, pH 7.5, 0.2% Tween 20, 1% bovine serum albumin). The membranes were then probed as before (4) with a monoclonal antibody against 3-nitrotyrosine (1:3,000 dilution; Upstate Biotechnology, Lake Placid, NY) for 2 h at 4 °C in blocking solution. The membranes were then washed five times in wash solution 1 and probed with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Amersham Biosciences) for 1 h at room temperature in blocking solution. After washing the membranes five times with wash solution 2 (20 mM Tris, 150 mM NaCl, pH 7.5), immunoreactive proteins were visualized on x-ray film using a chemiluminescent protein detection system (Amersham Biosciences). Nitrated BSA was used as a positive standard protein. X-ray film and gels were scanned with a GS-710 imaging densitometer (Bio-Rad), and the intensity of chemiluminescence and of Coomassie Blue staining was measured using PDQuest 2D Gel Analysis Software, Version 6 (Bio-Rad).

**Preparation of Nitrated BSA**—BSA was nitrated by incubating the protein (10 μg) with 50 μl tetranitromethane in 100 μl of 50 mM Tris-HCl, pH 8.0, at room temperature. After 5 min, the reaction was stopped by applying the preparation to a gel filtration column (Sephadex G-25, 1 × 5 cm; Pharmacia Biotech) equilibrated in 0.1% formic acid. The nitrated and desalted BSA was quantified by phenylthiocarbamyl amino acid analysis using an Applied Biosystems model 420H/130/920 automated analysis system (23). Nitrated BSA (30 μg) was added to 200 mM ammonium bicarbonate, pH 8, containing 8 M urea, diluted 1:4, and digested with 1 μg of trypsin (Promega, Madison, WI) at 37 °C overnight under argon.

**Protein Identification by MALDI-TOF Mass Spectrometry**—Identification of anti-nitrotyrosine reactive proteins by peptide mass map-
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**Fig. 1. Method for identification of nitrotyrosine-containing proteins.** Protein samples are separated in duplicate by 2D-PAGE and partially electrotransferred to PVDF membrane. One of the two membranes is treated with dithionite, and both are subjected to Western analysis with anti-nitrotyrosine antibody. Gels are stained with colloidal Coomassie Blue or Silver, and spots of interest are localized from the Western blots and excised for protein identification. Nitrotyrosine loses anti-nitrotyrosine antibody reactivity following reduction to aminotyrosine; therefore immunoreactive spots remaining after dithionite are false positives. See “Experimental Procedures” for details.

**Fig. 2. Western analysis before and after reduction of nitrotyrosine to aminotyrosine.** A retinal extract from a light-exposed 2-month-old rat (lanes 1 and 3, 20 μg/lane) and nitrated BSA standard (lanes 2 and 4, 20 ng/lane) were electrophoresed on 10% SDS-polyacrylamide gels, blotted to PVDF membrane, and probed with a monoclonal anti-nitrotyrosine antibody. Western analysis results are shown without (lanes 1 and 2) and after (lanes 3 and 4) membrane treatment with dithionite.

Peptides were extracted from in-gel digests with 60% acetonitrile containing 0.1% trifluoroacetic acid, dried in a Speed Vac, redissolved in 0.1% trifluoroacetic acid, and adsorbed onto C18 ZipTips (Millipore, Bedford, MA). Following ZipTip elution with 7 μl of 75% acetonitrile, 0.02% trifluoroacetic acid, peptides were subjected (1 μl) to mass spectrometric analysis using a Voyager DE Pro MALDI-TOF mass spectrometer (PE Biosystems, Framingham, MA). Measured peptide masses were used to query the Swiss Protein, TrEMBL, and NCBI sequence databases for matches using MS-Fit and Profound search programs. All searches were performed with a mass tolerance of 50 ppm. Positive identification required a minimum of five peptide matches and the highest probability ranking in both of the search programs employed.

**Protein Identification and Nitrotyrosine Analysis by LC MS/MS—LC MS was also used to analyze immunoreactive gel spots and nitrated BSA. Tryptic digests of gel spots (5–6 μl) were diluted with 0.1% formic acid (10 μl), and 10 μl were injected by autosampler onto a 0.3 × 1-mm trapping column (PepMap C18; LC Packings) using a CapLC system (Micromass), a switching valve (Micromass), and a flow rate of 5 μl/min. Solvent pumps were set at 8 μl/min, and the flow rate was controlled with a splitter in front of the switching valve. Peptides were eluted at 250 nl/min and chromatographed on a 50-μm × 5-cm Biobasic C18 column (New Objective, Cambridge, MA) with a gradient of 5–40% acetonitrile over 20 min followed by 80% acetonitrile for 5 min. The eluent was directed into a quadrupole time-of-flight mass spectrometer (QTOF2; Micromass, Beverly, MA) and ionized immediately using an electrosprayer designed in-house. The mass spectrometer was operated in standard MS/MS switching mode with the three most intense ions in each survey scan subjected to MS/MS analysis. In addition, the “include function” of the instrument operating software was used to program MS/MS analysis of precursor ions of all possible nitrotyrosine-containing tryptic peptides based on the structure of the proteins preidentified by MALDI-TOF MS (calculated as doubly and triply charged ions). Protein identifications and MS/MS data analyses utilized Micromass software ProteinLynx® Global Server, MassLynx® Version 3.5, and the Swiss-Protein and NCBI protein sequence databases (January 2002). MS/MS spectra of possible nitrated peptides were examined manually to determine sites of modification.

The relative amount of nitration at each nitration site in BSA was estimated based on the ion intensity of the nitrotyrosine-containing peptides, essentially as described for estimating relative amounts of
glycosylation (24). First the observed ion intensities for each charge state of a nitrated peptide were summed and then the total intensity for each nitrated plus unmodified peptide was calculated. The relative amount of nitration at the site was estimated as percent of the total intensity.

**Immunocytochemistry**—Eyes enucleated immediately after the light exposure were used for immunocytochemical analyses (25). After separating the anterior segment, posterior eye cups were fixed in 4% paraformaldehyde in PBS for 4 h at 4°C. The eyes were then cryoprotected in 30% sucrose overnight and embedded in optimal cutting temperature compound. Cryosections (16-μm) were prepared on gelatin-coated slides and stored at −80°C until use. Rats without light exposure were used as control animals. After air drying, sections were washed in PBS for 10 min and then incubated in PBS containing 5% BSA (Sigma) and 0.3% Triton X-100 for 1.5 h. The sections were then probed with mouse monoclonal anti-nitrotyrosine antibody (1:100 dilution; Upstate Biotechnology, Lake Placid, NY) in PBS containing 0.2% BSA and 0.2% Tween 20 overnight at 4°C. Fluorescein-conjugated anti-mouse IgG was used as secondary antibody (1:200 dilution, 1 h incubation at 4°C; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Sections were mounted with mounting medium (Vector Laboratories Inc., Burlington, CA), and normal mouse IgG was used as a control for the primary antibody. A total of 16 eyes (8 light-exposed and 8 control) were analyzed with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems Inc., Heidelberg, Germany). Images were captured from the posterior area of the retina, and more than 20 sections were examined per eye. The intensity of immunohistochemical reactivity was quantified using Quantity One software (Bio-Rad) after converting the color images from the confocal microscope to grayscale images in Adobe PhotoShop.

**RESULTS**

**Improved 2D Gel Western Detection of Nitrotyrosine-containing Proteins**—To distinguish between specific and non-specific nitrotyrosine immunoreactivity, we modified previously described methodology (4) and performed Western analyses before and after converting protein nitrotyrosine to aminotyrosine, as summarized in Fig. 1. Chemical reduction

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**FIG. 3. Sites of tyrosine nitration in tetranitromethane-modified BSA.** MS/MS spectra are shown identifying Tyr\(^{161}\), Tyr\(^{173}\), Tyr\(^{207}\), and Tyr\(^{224}\) as nitrated residues in tetranitromethane-modified BSA. A and E, BSA residues 161–167; B and F, BSA residues 169–183; C and G, BSA residues 347–359; and D and H, BSA residues 421–433. The spectra in A–D were from LC MS/MS analysis of 1 pmol of nitrated BSA digest. The spectra in E–H represent the lowest detectable amounts, namely (E) nY\(^{161}\), 5 fmol analyzed; (F) nY\(^{173}\), 75 fmol analyzed; (G) nY\(^{207}\), 250 fmol analyzed; (H) nY\(^{224}\), 5 fmol analyzed. Quantitative results are summarized in Table I. Calculated and observed precursor ions include doubly charged nitrated peptides m/z 486.7 (for nY\(^{161}\) peptide), m/z 806.9 (for nY\(^{207}\) peptide), and m/z 762.9 (for nY\(^{224}\) peptide) and triply charged m/z 645.3 (for nY\(^{173}\) peptide).
was achieved by treating PVDF-immobilized proteins with 10 mM sodium dithionite in 50 mM pyridine acetate, pH 5.0, at room temperature for 1 h. Before reduction, nitrated BSA exhibited strong nitrotyrosine immunoreactivity in Western analysis, and after reduction, the protein exhibited no immunoreactivity (Fig. 2, lanes 2 and 4). Western analyses of rat retinal proteins with and without dithionite reduction are also shown in Fig. 2 (lanes 1 and 3). Two bands, ~55 and 60 kDa, retain immunoreactivity after dithionite treatment, suggesting that these are false positives. Thus, Western analysis before and after dithionite reduction of membrane-immobilized proteins facilitates the identification of specific and nonspecific anti-nitrotyrosine antibody recognition.

Identification and Quantification of Nitration Sites in Chemically Modified Albumin—Albumin was modified chemically with tetranitromethane, and sites of nitration were identified and quantified by LC MS/MS analysis of tryptic peptides to evaluate the detection capabilities of our experimental approach. Analyses of the nitrated BSA tryptic digest yielded identification of four sites of tyrosine nitration, namely Tyr\textsuperscript{161}, Tyr\textsuperscript{173}, Tyr\textsuperscript{357}, and Tyr\textsuperscript{424} (Fig. 3). Unmodified peptides containing each of these residues were also detected. The relative amount of nitration at each site was estimated from four independent LC MS/MS analyses of 1–2 pmol of BSA tryptic digest to be ~84% at Tyr\textsuperscript{161}, ~44% at Tyr\textsuperscript{173}, ~5% at Tyr\textsuperscript{357}, and ~75% at Tyr\textsuperscript{424}. Detection sensitivity of nitrotyrosine-containing peptides was evaluated by LC MS/MS analysis of 1–1000 fmol of the nitrated BSA tryptic digest. The limits of detection for nitrotyrosine-containing peptides from this preparation of BSA were determined to be 4 to 33 fmol (see Table I, and see Fig. 3).

Identification of Anti-nitrotyrosine Reactive Proteins in Rat Retina Before and After Light Exposure—Retinas were isolated from a rat exposed to intense green light and another rat maintained in the dark, retinal proteins were fractionated by 2D gel electrophoresis, and nitrotyrosine-containing proteins were determined by Western analysis. A total of nine nitrotyrosine-immunopositive gel spots were detected in retinal extracts from control animals without light exposure (Fig. 4A), and 12 were detected from light-exposed animals (Fig. 4B). The immunoreactivity of spots 1 and 8 was significantly more intense in light-exposed than in control retina, whereas the intensity of spots 6, 7, and 9 appeared equal in both control and light-exposed retina. Following treatment with dithionite, spots 1, 9, 14, and 16 retained some immunoreactivity, suggesting that these are false positives (Fig. 4, C and D). Thus specific nitrotyrosine immunoreactivity appears to be associated with spots 2–5 in the dark, spots 10–13 and 15 following light exposure, and spots 6–8 in both control and light-exposed animals. The amount of immunoreactive protein remaining in the gel following partial electrotransfer was estimated to be ~15–274 ng per gel spot based on colloidal Coomassie Blue staining intensity (Table II).

Immunopositive protein spots were excised from the colloidal Coomassie Blue-stained gels (Fig. 4, E and F) and digested with trypsin, and peptides were analyzed first by peptide mass mapping using MALDI-TOF mass spectrometry and then by sequence analysis using LC MS/MS (Table II). MALDI-TOF MS provided single protein identifications for 15 of the 16 gel spots analyzed. LC MS/MS analysis confirmed the presence of all proteins identified by MALDI and identified additional proteins in 10 of the 2D gel spots. All nitrotyrosine-containing peptides were below detection limits. Proteins only identified from retina maintained in the dark were aldolase C, aldolase A, aspartate aminotransferase, glyceraldehyde-3-phosphate dehydrogenase, and annexin II. Proteins detected only from light-exposed retina were heat shock protein 70, dihydropyrimidinase-related protein 2, serum albumin, protein disulfide isomerase, glutamate dehydrogenase, and βA3/A1 crystallin. Additional proteins detected in gel spots that were immunopositive from both light-exposed and dark control retinas included heterogeneous nuclear ribonucleoprotein, voltage-dependent anion channel 1, malate dehydrogenase, βB3 crystallin, and three hypothetical/unknown proteins. Several other proteins were identified in spots that retain some immunoreactivity following reduction with dithionite. These proteins are considered potential false positives and include transducin β1, ATP synthase D, two hypothetical proteins, and βB2-, βA4- and γ-crystallin. Essentially the same results as in Fig. 4 and Table II were reproduced in identical analyses with a dark control rat and another light-exposed animal.

**Table I**

Detection of tyrosine nitration sites in tetranitromethane-modified BSA

| Amount analyzed (BSA tryptic digest) | Total peptides identified | Amount of nitrotyrosine peptide detected | Total peptides | Amount of nitrotyrosine peptide detected |
|------------------------------------|--------------------------|----------------------------------------|----------------|----------------------------------------|
| ng                                  | fmol                     | fmol                                   | ng             | fmol                                   |
| 66                                 | 1000                     | 22                                     | 840            | 440                                    |
| 17                                 | 250                      | 22                                     | 210            | 110                                    |
| 13                                 | 200                      | 20                                     | 168            | 88                                     |
| 5                                  | 75                       | 18                                     | 83             | 33                                     |
| 3                                  | 50                       | 15                                     | 42             | 15                                     |
| 0.7                                | 10                       | 8                                      | 8              | 8                                      |
| 0.3                                | 5                        | 7                                      | 4              | 4                                      |
| <0.1                               | 1                        | 1                                      |                |                                         |

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in the photoreceptor outer segments of animals maintained under dark conditions (Fig. 5A). Following light exposure, nitrotyrosine immunoreactivity almost disappeared in the photoreceptor outer segments but increased in the RPE and photoreceptor inner segments (Fig. 5B). Based on image analysis (Fig. 5D), the relative intensity of immunoreactivity increased ~20% in the RPE and photoreceptor inner segments and decreased ~90% in the photoreceptor outer segments after light exposure. Nitrotyrosine immunoreactivity in these regions of the retina was significantly above the background level immunofluorescence observed with normal mouse IgG (Fig. 5C). The outer plexiform layer of the retina also exhibited substantial levels of nitrotyrosine immunoreactivity with or without light exposure. Comparable results to those in Fig. 5 were obtained in immunocytochemical analyses with four light-exposed and four control rats.

Nitrotyrosine Immunoreactivity in Photoreceptor Outer Segments—Photoreceptor ROS were isolated from animals exposed to intense green light or maintained in the dark, and ROS proteins were compared by Western analysis using image analysis to quantify the intensity of nitrotyrosine immunoreactivity. With or without reduction with dithionite, the overall intensity of immunoreactivity in the ROS preparations from animals maintained under dark conditions was three to four times greater than that from light-exposed animals (Fig. 6A), supporting the results obtained by immunocytochemistry. Re-
### Table II

**Retinal proteins in nitrotyrosine immunoreactive gel spots**

| Gel spot | Amount (ng) | Protein | MALDI-TOF MS | LC-MS/MS | MW(kD)/pI | Accession number² |
|----------|-------------|---------|--------------|----------|-----------|-------------------|
|          |             |         | Peptide matches | Sequence coverage | Peptide sequences | Sequence coverage | Observed | Calculated |
| Proteins from dark control retinas |             |         |              |          |           |                   |          |
| 2        | 65          | Aldolase C | 9            | 41%      | 10        | 32%              | 41/7.2   | 39.2/6.8   | P09117   |
| 3        | 197         | Aldolase A | 7            | 36%      | 10        | 52%              | 41/7.8   | 39.3/8.3   | P05065   |
| 4        | 30          | Aspartate aminotransferase | 7 | 24% | 8 | 24% | 40/8.9 | 47.9/9.1 | P00507 |
| 5        | 274         | Heterogeneous nuclear ribonucleoprotein homolog 2 | 7 | 32% | 5 | 24% | 38/7.7 | 35.8/8.1 | Q90WU4 |
|          |             | Annexin II | 2            | 9%       | 2         | 9%               | 39/5.8   | 39.6/8.0   | P07959   |
| Proteins from light-exposed retinas |             |         |              |          |           |                   |          |
| 10       | 15          | Heat shock protein 70 | 9 | 18% | 15 | 28% | 60/5.6 | 70.2/5.6 | Q07439   |
| 11       | 30          | Dihydropyrimidinase-related protein-2 | 9 | 18% | 8 | 17% | 68.7/6.4 | Q16555 |
| 12       | 28          | Serum albumin | 8 | 17% | 8 | 17% | 68.7/6.4 | Q16555 |
| 13       | 30          | Protein disulfide isomerase ER60 | 9 | 20% | 17 | 33% | 52/6.0 | 56.6/6.4 | P11998   |
| 14       | 52          | Glutamate dehydrogenase | 5 | 13% | 15 | 32% | 47/7.0 | 61.4/8.1 | P10860   |
|          |             | β A3/A1 crystallin | 1 | 6% | 1 | 6% | 25.2/6.0 | P02525   |
|          |             | β A2/B1 crystallin | 7 | 53% | 7 | 46% | 27/6.2 | 25.2/6.0 | P02525   |
|          |             | β B2 crystallin | 1 | 6% | 1 | 6% | 23/4.6 | 23.4/6.5 | P26775   |
| Proteins in both dark control and light-exposed retinas |             |         |              |          |           |                   |          |
| 6        | 30          | Heterogeneous nuclear ribonucleoprotein A2/B1 | 10 | 30% | 10 | 41% | 36/8.0 | 36.0/8.7 | O85659   |
| 7        | 22          | Hypothetical protein XP_09339 | 2 | 8% | 2 | 8% | 33.7/5.6 | XP093339   |
|          |             | Voltage-dependent anion channel 1 | 2 | 8% | 2 | 8% | 33.7/5.6 | XP093339   |
| 8        | 19          | Heterogeneous nuclear ribonucleoprotein A2/B1 | 8 | 26% | 8 | 33% | 36/8.3 | 36.0/8.7 | O85659   |
|          |             | Malate dehydrogenase | 10 | 36% | 10 | 36% | 35.6/9.1 | P04636 |
|          |             | Unknown (protein for IMAGE:3615335) | 2 | 9% | 2 | 9% | 31.7/9.4 | AAH04945 |
|          |             | Hypothetical protein XP_09339 | 2 | 8% | 2 | 8% | 33.7/5.6 | XP093339   |
|          |             | Hypothetical protein | 5 | 32% | 7 | 38% | 29/7.0 | 34.3/6.7 | P02524   |
| False positives |             |         |              |          |           |                   |          |
| 1        | 146         | Transducin β chain 1 | 12 | 49% | 9 | 39% | 37/5.3 | 37.4/5.5 | P54311   |
| 9        | 148         | Hypothetical protein dJ1057B20.2 | 5 | 32% | 9 | 51% | 27/6.7 | 23.4/6.5 | P26775   |
| 14       | 38          | β A4 crystallin | 5 | 41% | 4 | 31% | 25/6.0 | 22.4/5.9 | P56374   |
|          |             | ATP synthase D chain | 1 | 9% | 1 | 9% | 16.6/5 | P31399 |
|          |             | Hypothetical protein | 1 | 1% | 1 | 1% | 118.8/6.3 | NP473081   |
| 16       | 43          | λ S crystallin | 3 | 10% | 26/6.8 | 20.8/7.4 | NP034097   |

a All identifications were from the 2D gels in Fig. 3. The identified species was rat unless the accession number is followed with (M) for mouse, (H) for human, or (P) for *Plasmodium falciparum*.
b Estimated amount based on colloidal Coomassie Blue staining intensity.
c Swiss Protein accession numbers are listed except those in italics, which refer to the NCBI database.
d Identified based on Blast search of the determined sequences.
duction with dithionite (Fig. 6B) resulted in a loss of nitrotyrosine immunoreactivity in ROS preparations from both dark-maintained (55% loss) and light-exposed animals (67% loss). The substantial loss of immunoreactivity, including that of nitrated standard protein BSA (Fig. 6B, lane 5), supports the presence of nitrotyrosine-containing proteins in ROS.

**DISCUSSION**

This study has sought improved methods of analysis for protein nitrotyrosine and evidence of a role for protein nitration in the mechanism of retinal light damage. Because immunological methods remain critical for the detection of nitration targets yet are subject to possible nonspecific antibody cross reactivity, we have utilized a 2D gel Western detection strategy that involves chemical modification of nitrotyrosine. Converting membrane-immobilized protein nitrotyrosine to aminotyrosine with dithionite (see Fig. 2 and Fig. 6) prevents specific recognition by anti-nitrotyrosine antibody (5). Anti-nitrotyrosine immunoreactivity remaining on the membrane after dithionite reduction is considered nonspecific. Dithionite reduction of nitrotyrosine-containing peptides to aminotyrosine analogs appears to be quantitative in peptides (26), and we suspect that for membrane-immobilized proteins, any nitrotyrosine accessible to antibody recognition will also be susceptible to modification by this small chemical reducing agent. Reduction of nitrotyrosine to aminotyrosine has been used to quantify nitrotyrosine in enzymatic and HCl protein hydrolysates using electrochemical, UV analysis and in mass spectrometric methods (3, 26, 27).

Although the dithionite reduction strategy improves the reliability of Western detection methods for nitrotyrosine, identification of nitrated proteins remains challenging, especially for low abundancy proteins in complex mixtures. Complications include antibody-inaccessible nitrotyrosine that remains...
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invisible by Western analysis and multiple potential components in a gel band or spot. The difference in MALDI-TOF mass spectra before and after dithionite treatment has been suggested as a method for identifying nitrotyrosine-containing peptides (26). However, none of our MALDI-TOF MS analyses of 2D gel spots or of 2 pmol of nitrated BSA tryptic digest exhibited the laser-induced decomposition products reported as possible nitrotyrosine signature ions (26), presumably because of insufficient sample amounts. Collision-induced dissociation in electrospray tandem mass spectrometry produces an immonium ion \(m/z\) 181.06) from nitrotyrosine that has been suggested as useful for precursor ion scanning to identify \textit{in vivo} sites of nitration (28). Our attempts to monitor this immonium ion with the QTOF2 instrument were not informative. We also pursued precursor ion scanning of the immonium ion using nitrated BSA tryptic digest (2 pmol) and a triple quadrupole electrospray instrument (Sciex API 3000). A couple of nitrated albumin peptides were detected, but the false positive signals described previously (28) were problematic, and the detection sensitivity was insufficient for productive analysis of the rat retinal samples.

By programming the QTOF2 mass spectrometer to perform MS/MS on all possible nitrotyrosine peptides in BSA regardless of ion intensity, Tyr\(^{161}\), Tyr\(^{173}\), Tyr\(^{357}\), and Tyr\(^{424}\) were identified as sites of nitration (Fig. 3) with detection limits in the 4 to 33 fmol range (Table I). Several other studies using albumin modified with either tetranitromethane or peroxynitrite also identified nitration at Tyr\(^{161}\) (Tyr\(^{162}\) in human serum albumin) and/or Tyr\(^{324}\) (26, 28, 29), the most extensively modified sites in the present study. Other tyrosines reported to be nitrated in \textit{in vitro}-modified BSA include Tyr\(^{54}\), Tyr\(^{163}\), Tyr\(^{475}\) (28), and Tyr\(^{364}\) (26) and in human serum albumin, Tyr\(^{435}\) (29). Clearly the nitrating agent and reaction conditions can influence the extent of chemical modification; however the factors determining the selectivity of tyrosine nitration remain unclear. For example, we observed only one of the four tyrosines nitrated in BSA peptide HPYFn\(\text{APELLYYANK}\) (residues 169–183). Limitations of this detection strategy are that the identity of the nitrated protein must be known in advance, and nitrated peptides with additional, unpredicted post-translational modifications will not be detected.

The nitrotyrosine peptide detection strategy used here appears appropriate for methods development studies of model proteins like BSA; however additional sensitivity is required for the routine identification of nitration sites in \textit{in vivo} samples. The average 2D gel spot in the present study of rat retinal proteins (Fig. 4) contained about 70–80 ng of protein and multiple components (Table II). Allowing \(~30\%\) loss during post-gel sample preparation (30) and \(~14\%\) consumption for MALDI-TOF MS, the average amount analyzed by LC MS/MS per gel spot was \(~40–50\) ng. No nitrotyrosine was detected in the retinal samples by mass spectrometry although the analysis strategy allowed identification of two sites in BSA with only \(~0.3\) ng of tryptic digest (Table I). Greater sample amounts, enrichment methods, and/or higher sensitivity will be needed to identify nitration sites in the retinal samples.

Many of the retinal proteins exhibiting nitrotyrosine immunoreactivity in this study were also detected with anti-nitrotyrosine antibody in liver or cultured cells following inflammatory challenge (4). Although sites of nitration have not been identified, the present results nevertheless indicate different nitration events have occurred in retinal tissue from light-exposed rats compared with animals maintained in the dark (Table II). Of the five proteins only identified in rats maintained in the dark, four function in energy production, namely fructose biphosphate aldolase A and C, aspartate aminotransferase, and glyceraldehyde-3-phosphate dehydrogenase. Reinforcing the presence of nitrotyrosine in aldolase was the identification of this protein by LC MS/MS in two different dithionite-sensitive, immunoreactive gel spots and that no other proteins were present (see Fig. 4, and see Table II). This provides evidence of tyrosine nitration under \textit{in vivo}, non-pathological conditions and suggests protein nitration plays a role in normal retinal physiology. Among the unique proteins only identified in rats exposed to intense light were mitochondrial enzyme glutamate dehydrogenase, stress response proteins HSP 70, protein disulfide isomerase, \(\beta A3/A1\) crystallin, and serum albumin, which carries photolabile S-nitroso aducts (31). Protein disulfide isomerase and heterogeneous nuclear ribonucleoprotein A2/B1, a nuclear RNA-binding protein of unknown function, were each identified by LC MS/MS as the sole component of nitrotyrosine-immunoreactive gel spots following light exposure (Table II). The \textit{in vivo} consequences of these nitration events are not clear; however \textit{in vitro} studies have shown that nitration of tyrosine can inhibit the activity of three of the identified enzymes, namely for aldolase (32), aspartate aminotransferase (33), and glutamate dehydrogenase (34). Although we do not know whether all of
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the proteins in the immunoreactive spots contain nitrotyrosine, the results demonstrated that retinal proteins are nitrated in the dark and that exposure to damaging light changes the nitration targets.

Our immunocytochemical results also showed that the nitration targets within the cell layers of the rat retina changes with light exposure. More tyrosine nitration appears in the photoreceptor outer segments in the dark (Fig. 5) where endogenous NO produced by NO synthase is a likely contributing factor. Endogenous NO has been linked functionally to guanylyl cyclase activity in photoreceptor cells (35), suggesting a possible modulatory role for NO in visual transduction. The present results raise questions as to whether nitration in photoreceptor outer segments might participate in modulating disc membrane renewal in the dark and the rhythmic light/dark regulation of disc shedding at day break. Increased tyrosine nitration was observed in the photoreceptor inner segments and RPE following intense light exposure, apparently because of increased concentrations of reactive nitrogen oxide species. This mechanism for modification seems particularly plausible in the inner segments of the photoreceptors where a major cellular source of reactive oxygen species are concentrated, namely the mitochondria (4). Following light exposure, the decrease in photoreceptor outer segment nitrotyrosine observed by both immunocytochemical (Fig. 5) and Western (Fig. 6) analyses may in part reflect oxidative stress-induced selective protein degradation by the proteasome (36).

Nitrotyrosine is formed from the reaction of free or protein-bound tyrosine with reactive nitrogen oxide species. The actual species responsible for tyrosine nitration in vivo continue to be debated; however free radical nitrogen dioxide ($NO_2^-$) (37) and peroxynitrite (ONOO$^-$) (38) have both been implicated as major contributors. Nitrogen oxide species also react with cysteine thiols in vivo (39), and in plasma a majority of the total NO (e.g. 84%) appears to circulate as S-nitroso adducts of serum albumin (31). Glutathione may also serve as a source of both nitrating and cytotoxic reactive nitrogen oxide species.

We have employed a well established in vivo model system for light damage that typically results in 50% photoreceptor cell loss in rats within 2 weeks of the light exposure (8, 10). In the present study, animals were sacrificed immediately after light treatment to probe for initial light-induced changes in protein nitration. Clearly, the cytotoxic effects of nitric oxide (42) may also be manifested in retinal light damage. Notably, excessive nitric oxide has been implicated in retinal damage in rat models of ocular ischemia (43) and chronic glaucoma (44). Excess reactive nitrogen oxide species not only modify proteins, lipids, and DNA, but in the retina may also contribute to the oxidative modification of retinoids in the pathways of light-induced retinal damage. The present findings justify further consideration of nitration and nitric oxide as possible mediators in light-induced photoreceptor cell death.

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