Light Exposure Induces Ubiquitin Conjugation and Degradation Activities in the Rat Retina

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Purpose. To evaluate the consequences of light exposure on retinal ubiquitin (Ub) conjugation and degradation.

Methods. Two-month-old Long Evans pigmented rats were exposed to constant light (180 foot-candles) or were left in complete darkness for 18 hours. Rats used for cyclic light and diurnal rhythm experiments were removed from their light cycles at different times (24-hour clock): 0700 (before the light was turned on), 1000 (3 hours into the light cycle), 1000D (continued in the dark cycle), 1900 (before the light was turned off), 2200 (3 hours into the dark cycle), and 2200L (continued in the light cycle). The retinas were examined for Ub conjugation, adenosine triphosphate-Ub-dependent degradation, levels of Ub messenger RNA, and localization of Ub immunocytochemistry.

Results. There was a statistically significant increase in Ub conjugation and degradation in retinas isolated from light-exposed animals compared with degradation in retinas of dark-adapted animals. However, no significant differences were observed in the levels of Ub messenger RNA from cyclic light, or light-exposed or dark-adapted retinas, suggesting that light-stress-induced changes do not reflect increased transcriptional activity. The daily variations observed in Ub conjugation and degradation suggest that these processes are probably the result of a circadian rhythm. Results of immunohistochemical studies revealed that Ub and its conjugates were uniformly distributed throughout the retinal cell layers in light- and dark-adapted retinas. However, in light-exposed retinas, a strong positive immunoreactivity was observed in the inner retina, specifically in horizontal and ganglion cells.

Conclusions. These results suggest that light exposure may play a role in inducing Ub-conjugating activity in certain retinal cells. Furthermore, the results support the hypothesis that Ub is a stress protein that plays an important role in protecting cells under stress conditions. Invest Ophthalmol Vis Sci. 1997; 38:2344-2354.

Retinal photoreceptors are neural cells devoted to the transduction of light signals into electrical signals. Absorption of a photon by a single molecule of the visual pigment rhodopsin is sufficient to alter membrane conductance, resulting in hyperpolarization and a transient decrease in photoreceptor dark current. Although photoreceptors are highly specialized for phototransduction, their prolonged or excessive exposure to light can lead to irreversible damage. The extent of damage caused by constant illumination is affected by various factors. Among these are species, history of light exposure, age, diet, genetic makeup of the animal model, and properties of the incident light, including intensity, duration, and wavelength. Additionally, susceptibility of the retina to light damage is influenced, to some extent, by dark-adapted and steady-state levels of rhodopsin before and during light exposure.

In several studies it has been suggested that lipid peroxidation plays a causal role in retinal light damage. In the vertebrate retina is known to contain several defense systems that reduce the harmful effects of lipid peroxidation. Among these are antioxidant enzymes (glutathione enzymes and superoxide...
ATP, subsequently transferring the activated Ub from dismutase)\(^6\) and other natural antioxidants (glutathione and vitamins E and C).\(^6,^{14}\) Levels of some of these antioxidants become elevated in retinas of rats reared in high-intensity cyclic light.\(^6,^{16}\)

Hyperthermia, as a stress, increases the levels of several heat shock proteins (HSPs) in the rat retina.\(^{15,16}\) Induction of hyperthermia before light exposure reduces the degree of photoreceptor photic damage, presumably because of the elevated level of HSPs.\(^{15,16}\) The intracellular levels of ubiquitin (Ub) and its conjugates change in response to heat shock.\(^17\) Because Ub has been identified as a heat-shock- and stress-regulated protein in several kinds of cells,\(^{18-21}\) it is possible that Ub plays a similar role in light-stressed retinas. In fact, the notion of the protective role of Ub in stressed retinas is supported by experiments showing that such stresses as hyperthermia, ischemia, and trauma increase the expression of Ub and HSPs in brain tissues.\(^{22,23}\)

Ubiquitin is a 76-residue protein that can be attached covalently to a number of cytoplasmic, nuclear, and integral membrane proteins.\(^24\) Ubiquitination is known to facilitate adenosine-triphosphate (ATP)-dependent degradation of targeted proteins by a complex protease called the 26S proteasome.\(^25\) Ubiquitin-dependent proteolysis has been implicated in a variety of cellular processes, including stress response,\(^{20,26}\) cell-cycle control,\(^27\) DNA repair,\(^28\) and apoptosis.\(^29\) Ubiquitin is also known to play a role in promoting cell survival during the metabolic alterations that occur in stress.\(^21\) The formation of Ub–protein conjugates involves a cascade of enzymatic reactions that are initiated by the Ub-activating enzyme (E1), activating the C-terminal glycine of Ub by ATP, subsequently transferring the activated Ub from E1 by a Ub-conjugating enzyme (E2) to l-lysyl e-NH\(_2\) groups on the protein substrate. Alternatively, E2 may transfer the activated Ub from E1 to a substrate that is bound to a Ub–protein ligase (E3), which then catalyzes the last step in the conjugation process.

Ubiquitin conjugates can also undergo disassembly by the action of Ub–carboxyl-terminal hydrolase.\(^30\) In this ATP-independent process, Ub moieties are removed from the conjugate, releasing free substrate and Ub.\(^24\) Therefore, in vivo, the level of Ub conjugates results from a balance between the rates of conjugate formation, dissociation, and degradation. A breakdown in this balance has been shown to occur in certain neural diseases, including Alzheimer’s.\(^8,^{21}\)

Previously, we demonstrated the presence of Ub and its conjugating system in the rat retina.\(^32\) More than 50% of retinal Ub is covalently bound to proteins. The retina\(^32\) and more specifically the photoreceptor cells\(^33\) seem to have highly active and stable Ub-conjugating and degrading activities, compared with those of the reticulocyte system. Several photoreceptor-specific proteins (rhodopsin\(^33\) and transducin\(^34\)) have been shown to be ubiquitinated in vivo and in vitro. The objectives of the present study were to determine whether light stress has an effect on Ub conjugation and degradation, determine the effect of light on Ub localization within the retina, and examine the daily variations in the steady-state levels of retinal Ub and its conjugates. During the course of these experiments, the intensity and duration of light exposure were chosen only to stress the retinas without causing the permanent photic damage known to occur in rats.\(^2\)

Our data indicate that Ub plays an important role in protecting the retina from light stress. Most significant, we show a higher level of Ub immunoreactivity with light stress in the inner retina, in the region where horizontal and ganglion cells are located. Our results also suggest that Ub-related processes are active in the retina and that the degradation and subsequent disposal of retinal proteins may be regulated by a circadian rhythm.

**METHODS**

**Antisera**

Polyclonal rabbit antibody against sodium dodecyl sulfate (SDS)-denatured human Ub (conjugated to γ-globulin) was generated and purified by affinity chromatography, using a column containing the corresponding Ub immobilized on Affi-Gel 10 (Bio-Rad, Hercules, CA).\(^35\) The antibody was concentrated, and the buffer was exchanged by ultrafiltration with a CF-25 Centriflo membrane (Amicon, Beverly, MA) into 50 mM phosphate-buffered saline (pH 7.4) containing 5 mM EDTA. The recovery of antibody titer using this procedure was around 80%. Similar immunoreactivity was observed with this antibody when compared with anti-Ub antibody (provided by Dr. Arthur Haas, Medical College of Wisconsin, Milwaukee).\(^35\) For immunoblot and immunocytochemical analyses, free and conjugated Ub were detected using diaminobenzidine, as previously described.\(^35\)

**Light Treatments**

Two-month-old Long Evans rats weighing 180 to 200 g were obtained from Charles River (Wilmington, MA) and housed under a cyclic light environment of 25 foot-candles (fc) illumination for 12 hours/day (lights on at 0700 and off at 1900). Rats were adapted to this light regimen for a minimum of 1 month before they were used for experiments.

In one set of experiments, rats were removed 3 hours after the start of their light cycle (at 1000) and kept in a temperature- and humidity-regulated incubator for 18 hours of complete darkness or constant light exposure of 180 fc. The duration of light exposure was based on the observation that HSPs are induced in rat retinas 18 hours after hyperthermia begins.\(^15\)
Assay of Retinal Ubiquitin Conjugation

A total of 2 measurements were done under dim red light. Experiments were used immediately. Retinas from dark-adapted rats were removed from their light cycles at different intervals around the time of light on (0700) and light off (1900). During the light period, four rats were removed at 0700 (before lights-on); at 1000 (3 hours in the dark cycle); and at 1000D (continued in the dark without entering the light cycle). During the dark period, four rats were removed at 1900 (before lights-off); at 2200 (3 hours in the dark cycle); and at 2200L (continued in the light without entering the dark cycle). Retinas from all rats in the group were pooled and processed, as described later, and for Ub conjugation and degradation to determine the steady-state levels of Ub and its conjugates in the retina during the light cycle. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Retinal Samples

For each experiment, four to eight rat retinas were combined and processed as one sample. The retinas were homogenized at approximately 1600 rpm in 50 mM Tris-HCl buffer (pH 8), and 10 mM MgCl₂, using a motor-driven Teflon pestle with a 0.004- to 0.006-mm clearance. The resulting retinal homogenates were used immediately. Retinas from dark-adapted rats were dissected and homogenized, and all the measurements were done under dim red light. Experiments were repeated four to eight times for statistical evaluations.

Assay of Retinal Ubiquitin Conjugation

Conjugation of Ub to retinal proteins was performed as has been described previously. The measurements were made in the light for the light-exposed samples and in the dark for the dark-adapted samples. Reactions were performed in the presence of an ATP-regenerating system (2 mM ATP, 20 mM creatine phosphate, and 0.5 U creatine phosphokinase) or an ATP-depleting system (50 mM 2-deoxyglucose and 2 U hexokinase). In some experiments, EDTA (2 mM) was used to eliminate ATP instead of the ATP-depleting system. Retinal homogenates (100 to 150 μg total protein) from light- or dark-treated animals were used as a source for Ub-conjugating enzymes and substrates. A total of 2 μg of iodine-125 ([¹²⁵I]-Ub) (10⁶ cpm) was used in each assay. Four sets of controls were carried out with each sample: presence of [¹²⁵I]-Ub alone (background; control 1), absence of Ub and retinal homogenates (control 2), absence of Ub (control 3), and absence of retinal homogenate (control 4). The counts from controls 2, 3, and 4 were similar to that from the background (control 1). The average value of these three controls was subtracted from the counts measured in the ATP-supplemented and ATP-depleted reactions of each sample. Furthermore, reticulocyte lysate was used for comparison. The reactions were incubated at 37°C for 15 minutes and were terminated by denaturation with SDS sample buffer, and the solubilized proteins were separated by 7.5% to 20% SDS–polyacrylamide gradient gel electrophoresis (SDS–PAGE).

In pulse–chase experiments, retinal samples were pulsed with [¹²⁵I]-Ub for 15 minutes at 37°C and then chased with a 100-fold molar excess of unlabeled Ub for 30 minutes. Aliquots containing approximately 10⁶ cpm were separated during SDS–PAGE. The gels were silver stained, photographed, and dried; and the location of radioactivity in the free and conjugated forms of [¹²⁵I]-Ub was determined by autoradiography. The lanes related to Ub conjugates were cut out and counted in a γ-counter. The pulse counts represented the levels of Ub-conjugating activities in the retinas from light- and dark-adapted animals. The chase counts represented the levels of conjugates that resisted ATP- and Ub-dependent degradation or disassembly during the 30-minute chase. The difference between the two illustrated the level of conjugates eliminated during the chase by ATP- and Ub-dependent degradation or disassembly by Ub–carboxyl-terminal hydrolases.

Assay of Retinal Ubiquitin-Dependent Degradation

The breakdown of radiolabeled, reduced, and carboxymethylated bovine serum albumin (¹²⁵I-rcmBSA) to acid-soluble material was determined as described previously. The reaction mixture in its final volume of 50 μl contained 50 mM Tris-HCl buffer (pH 8), 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 2 mM ATP, 20 mM creatine phosphate, 0.5 U creatine phosphokinase, 5 μg Ub, 1 to 2 μg ¹²⁵I-rcmBSA (10⁶ cpm), and 90 μg of retinal homogenate prepared from light- or dark-adapted retinas. After incubation at 37°C for 1 hour, the reactions were terminated by the addition of 50 μl BSA (11.5 mg/ml) and 0.9 ml of 12.5% trichloroacetic acid (TCA). The release of TCA-soluble material was determined by γ-counting.

The same sets of controls were carried out with each sample as described previously and indicated earlier for the Ub-conjugation assay. The acid-soluble counts in these controls were similar to that in the background. The acid-soluble counts in ATP-depleted samples, which represent the ATP-independent degradation, were subtracted from the counts measured in the ATP-supplemented samples. The subtracted values represented the actual degradation of ¹²⁵I-rcmBSA by retinal ATP- and Ub-dependent proteases. These values were expressed as counts per minute per milligram of total retinal protein.
Northern Blot Analysis

Retinas from rats adapted to various light treatments were extracted and immediately dropped into liquid nitrogen, and messenger RNA (mRNA) was isolated, using a FastTrack kit (Invitrogen, San Diego, CA). Then mRNA was separated by electrophoresis on 1% agarose gel, containing 2.2 M formaldehyde and transferred by capillary action to Zetaprobe (Bio-Rad) or Nytran (Schecher and Schuell, Keene, NK) nylon membranes. The RNA blots were hybridized with a 32P-labeled human Ub genomic fragment,37 mouse opsin genomic fragment,38 or actin cDNA clone in a solution containing 50% formamide and 6 X SSC (0.90 M NaCl, 0.09 M sodium citrate) or 1.5 X SSPE (0.36 M NaCl, 0.02 sodium phosphate, pH 7.7, and 0.002 mM EDTA) at 42°C. The filters were sequentially washed for 1 hour in the same solution as that used for the washes. The secondary antibody was applied for 1 hour in 0.15 M NaCl with 0.1 M Tris-HCl (pH 7.6) and 0.002 mM EDTA) at 42°C. The filters were sequentially washed for 1 hour in 0.15 M NaCl with 0.1 M Tris-HCl (pH 7.6), and the secondary antibody was omitted. No radioactive bands were observed in the autoradiographs, which contained ATP and an ATP-regenerating system.

Immunocytochemical Analysis

For immunohistochemical detection of Ub, eyes were enucleated from 18 hours light- or dark-adapted rats for 18 hours. The cornea and lens were removed, and the eye cups were fixed in 4% paraformaldehyde, 3% sucrose, and 0.1 M sodium phosphate (pH 7.2) at 4°C overnight. The retinas were then washed in 3% sucrose, 0.1 M sodium phosphate (pH 7.2) for at least 1 hour and cryoprotected in 30% sucrose with 0.1 M sodium phosphate (pH 7.2). Transverse frozen sections were cut at 12 to 16 μm with a cryostat, placed on warm gelatin-coated slides, dried at 24°C, and stored at −20°C.

Before application of the primary antibody, sections were preincubated for 45 minutes in blocking solution containing 2.5% BSA, 2% normal goat serum, 0.3% Triton X-100, 0.15 M NaCl, and 0.1 M Tris-HCl (pH 7.6). Sections were incubated at 4°C overnight in a humidified chamber with various concentrations of anti–Ub-antibody in 0.1 ml of the blocking solution (1:100 to 1:10,000 dilution). No differences in staining were observed in the dilution of the primary antibody between 1:1,000 and 1:10,000. Subsequent steps were carried out at room temperature. Slides were washed for 1 hour in 0.15 M NaCl with 0.1 M Tris-HCl (pH 7.6), and the same solution was used for subsequent washes. The secondary antibody was applied for 1 hour in the blocking solution, except that 0.02% Triton X-100 was added. The tissues were washed for 45 minutes, and peroxidase antiperoxidase was applied for 1 hour in the same solution as that used for the secondary antibody. The tissues were washed for 20 to 30 minutes, and the peroxidase antiperoxidase was treated with 1 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), 0.01% H2O2, and 0.05 M Tris-HCl (pH 7.6) for 5 to 10 minutes. After a brief wash, the sections were dehydrated through graded ethanol to xylene and mounted in Permount (Fisher Scientific, Itasca, IL).

Control sections were observed in the levels of some Ub-protein samples were observed in the levels of some Ub–protein

Statistical Analysis

Results were reported as mean ± SEM and were compared by analysis of variance. Statistical significance was considered to be P < 0.05.

Other Methods

Ubiquitin was labeled with 125I by chloramine-T39 or as described by Bolton and Hunter40, both preparations of labeled Ub were identical in their conjugation and degradation activities. The preparation of recombinant (r)BBA was carried out as described before.39 Protein concentration was determined by the method of Bradford,41 using BSA as a standard.

RESULTS AND DISCUSSION

Metabolism of Ubiquitin Conjugates in Light-Stressed and Dark-Adapted Retinas

Conjugation and degradation of Ub were measured in retinal samples from rats that were exposed to constant light or left in complete darkness for 18 hours (Fig. 1). The conjugation activity was measured by the incorporation of 125I–Ub into retinal proteins in the presence of ATP and an ATP-regenerating system (Fig. 1B). Radiolabeled conjugates, detected by autoradiography, were observed only in conjugation assays that contained ATP and an ATP-regenerating system. No radioactive bands were observed in the autoradiograms when the ATP-depleting system or EDTA was used (data not shown, see Fig. 1B of Naash et al).35 In light-exposed retinal homogenates, the level of high-molecular-weight (HMW) Ub conjugates (above 50 kDa) increased compared with the level in the dark-adapted retinal samples (Fig. 1B, lanes 3 and 4, compared with lanes 1 and 2). This increase was significant, because no obvious differences were seen in the amount of protein loaded in the comparable lanes (Fig. 1A, compare lanes 3 and 4 with lanes 1 and 2). Furthermore, no differences between light and dark samples were observed in the levels of some Ub–pro
FIGURE 1. Light exposure increases the levels of ubiquitin conjugation and degradation in the rat retina. Ubiquitin conjugation reactions were performed on different samples of retinal proteins from dark-adapted (lanes 1 and 2) and light-exposed (lanes 3 and 4) rats. The conjugation reactions, 125I-labeled reduced and carboxymethylated bovine serum albumin (rcmBSA) proteolysates, and the light treatments were as described in the Methods section. Retinas from rats were isolated after 18 hours of dark adaptation or light exposure and then homogenized. For conjugation reactions, homogenates (150 μg of total proteins) were incubated at 37°C for 15 minutes with 2 μg 125I-Ubiquitin (10^6 cpm) and 2 mM adenosine triphosphate with an adenosine triphosphate-regenerating system. The reactions were stopped by adding 2 × Laemmli sample buffer. The samples were run on 7.5% to 20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). (A) The gel was silver stained, and (B) ubiquitin conjugation was visualized by autoradiography. (C) For in vitro proteolysis of 125I-reduced and carboxymethylated bovine serum albumin to acid-soluble material, retinal homogenates (90 μg of total proteins) from light-exposed or dark-adapted rats were incubated at 37°C for 1 hour in the presence of 2 mM adenosine triphosphate, adenosine triphosphate-regenerating system, and 5 μg ubiquitin. The reactions were terminated by the addition of 50 μl of 11.5 mg/ml bovine serum albumin and 0.9 ml 12.5% trichloroacetic acid. The samples were centrifuged for 10 minutes, and 0.8 ml of the supernatant was counted for radioactivity. Controls without adenosine triphosphate (in the presence of 2 mM EDTA) were also included, and the acid-soluble radioactivity present at 0 time incubation was subtracted. Results from six samples were expressed as acid-soluble counts per minute per milligram of protein (cpm/mg protein) from samples treated in light (L) or dark (D).

Our results suggest that exposure to bright light increases synthesis and degradation of retinal Ub conjugates. These data also raise the fundamental question of whether the light effect on Ub reflects an increase in the availability of Ub substrates, in the stimulation of Ub-conjugating enzymes, or in both. It is possible that light exposure alters some retinal proteins, which become better substrates for Ub conjugation. Whether light-induced Ub-conjugating activity or the level of proteins that are susceptible to Ub conjugation has any specific physiological function in the retina during light stress is not known. However, similar to its role in other systems, 20'21'23 it is possible that Ub plays a role in modulating the effects of light stress on the retina.

Ubiquitin Transcripts in Light-Stressed and Dark-Adapted Retinas

Many metabolic functions are triggered when the retina is exposed to light, 44'45 some of which depend on the translation of preexistent mRNA or synthesis of

Adenosine triphosphate with an adenosine triphosphate-regenerating system from light-exposed or dark-adapted rats were incubated at 37°C for 1 hour in the presence of 2 mM adenosine triphosphate, adenosine triphosphate-regenerating system, and 5 μg ubiquitin. The reactions were stopped by adding 2 × Laemmli sample buffer. The samples were run on 7.5% to 20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). (A) The gel was silver stained, and (B) ubiquitin conjugation was visualized by autoradiography. (C) For in vitro proteolysis of 125I-reduced and carboxymethylated bovine serum albumin to acid-soluble material, retinal homogenates (90 μg of total proteins) from light-exposed or dark-adapted rats were incubated at 37°C for 1 hour in the presence of 2 mM adenosine triphosphate, adenosine triphosphate-regenerating system, and 5 μg ubiquitin. The reactions were terminated by the addition of 50 μl of 11.5 mg/ml bovine serum albumin and 0.9 ml 12.5% trichloroacetic acid. The samples were centrifuged for 10 minutes, and 0.8 ml of the supernatant was counted for radioactivity. Controls without adenosine triphosphate (in the presence of 2 mM EDTA) were also included, and the acid-soluble radioactivity present at 0 time incubation was subtracted. Results from six samples were expressed as acid-soluble counts per minute per milligram of protein (cpm/mg protein) from samples treated in light (L) or dark (D).
TABLE I. Effect of Light Exposure on Retinal-Ubiquitin (Ub)-Protein Conjugates

<table>
<thead>
<tr>
<th>Light Exposure</th>
<th>Total Ub Conjugates (cpm/mg protein) or % Incorporated or Degradated</th>
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<tbody>
<tr>
<td><strong>Pulse (15 min)</strong> (total conjugate counts)</td>
<td>26,065 ± 1769 (100%)</td>
</tr>
<tr>
<td><strong>Chase (30 min)</strong> (% of counts remaining)</td>
<td>13,096 ± 3505 (50%)</td>
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<tr>
<td><strong>Counts lost in the chase</strong> (% of counts lost)</td>
<td>12,969 ± 3427 (50%)</td>
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* Retinal 125I-Ub conjugates were prepared as described in the text. Retinal samples (150 μg of protein) were incubated at 37°C for 15 minutes with 2 μg of 125I-Ub (10^6 cpm) and 2 mM ATP and either ATP-regenerating system or ATP-depleting system. At the end of the incubation, samples were divided into two parts: in one part, the reaction was stopped by adding 2X Laemmli sample buffer, whereas in the other part, a 100-fold molar excess of unlabeled Ub was added and the reaction was continued for 30 minutes at 37°C. The samples from the pulse and the chase were run on 7.5% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Ub conjugates were visualized by autoradiography of the dried gels, and the lanes representing these conjugates were cut and counted for both the pulse and the chase. Data are a combination of four to eight independent retinal samples, presented as mean ± SD.

new mRNA after light exposure. Moreover, results of studies have shown that Ub and its mRNA are induced in several tissues under different types of stress. To determine whether the steady state levels of Ub transcripts are modulated by constant light exposure or dark adaptation, we performed RNA blot analyses. We analyzed the size and complexity of mRNAs encoding Ub in poly(A)+ RNA that was extracted from retinas of rats that had been in the light cycle for 3 hours (1000), in complete darkness, or in constant light exposure of 180 fc for 18 hours (Fig. 2, lanes A, B, and C, respectively). The RNA samples were probed with a 32P-labeled human poly-Ub cDNA fragment, containing the entire Ub coding region. The resulting hybridization pattern is shown in Figure 2A. The human-labeled cDNA fragment hybridized to two mRNAs of estimated sizes 1.4 kb and 3.5 kb in retinal RNAs after light or dark treatment (Fig. 2A). The 1.4-kb transcript was the predominant Ub transcript observed in the rat retina. Both of these retinal transcripts, along with a 2.8-kb message, were also observed in the rat brain (results not shown). Four Ub transcripts of estimated sizes 670 bp, 1.5 kb, 1.6 kb, and 3 kb have been previously detected in rat brain and other tissues (testis, pancreas, and thymus). However, we were not able to detect the 670-bp and 1.5-kb transcripts for Ub in RNA preparations from retina or brain. Our 1.4-kb band was equivalent to the already documented 1.6-kb rat brain Ub transcript. The same applied to our observed 2.8-kb transcript and the documented 3-kb transcript. Regarding the absence of the 760-bp and 1.5-kb transcripts, we believe that they are products of partial degradation of larger poly-Ub mRNA or that they represent strain or tissue differences in mRNA species for Ub.

Characterization of the rat retinal Ub mRNA by nucleotide sequence analysis of cDNAs will establish the identity of these transcripts and will determine the existence of poly-Ub precursors in these species. This information will also provide a basis on which to identify retina-specific Ub transcripts and to investigate

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933419/ on 06/04/2018)
whether these transcripts are the products of different genes.

The RNA blot shown in Figure 2A was stripped and probed with human β-actin (Fig. 2B) and mouse opsin sequences (Fig. 2C). Quantification of the autoradiographs showed that the steady-state levels of Ub transcripts in the retinal cell layers from dark-adapted rats (Fig. 5B). These strongly immunoreactive cells at the junction of the inner nuclear layer (INL), and in the ganglion cell layer (GCL) (Fig. 5A, open arrows). These strongly immunoreactive cells at the junction of the inner nuclear layer (INL), and in the ganglion cell layer (GCL) (Fig. 5A, open arrows).

Cyclic Light and Diurnal Rhythm Effect on the Metabolism of Ubiquitin Conjugates

Many retinal events are induced by the onset of light. For example, most of the daily new membrane is added to the outer segment during the first 8 hours of the light cycle. Hollyfield and Basinger showed that RNA synthesis is highest during the early light phase, whereas protein and glycoprotein synthesis are highest during the dark phase of the cycle. With this evidence of daily variation in rates of membrane addition and several other aspects of metabolism, we were interested in determining whether Ub conjugation and degradation undergo changes during a 24-hour cycle. Therefore, we used 125I–Ub to observe the levels of its incorporation into retinal proteins of rats maintained for at least 1 month at a diurnal cycle of 12 hours of light and 12 hours of darkness.

Pulse–chase experiments with 125I–Ub were performed on whole retinal extracts isolated from rats at different times during cyclic light (0700, 1000, 1900, and 2200; Fig. 3A). The pulse and the chase samples were resolved in parallel by SBD–PAGE and analyzed by autoradiography. Quantification of the data from four independent experiments is shown in Figure 3B for Ub conjugates and in Figure 3C for ATP- and Ub-dependent degradation of the conjugates. The data indicate that there is a daily, related change in Ub conjugation and Ub degradation. However, conjugate levels increased to a maximum at 0700, before the light came on, and declined significantly during the light cycle (P < 0.035). Figure 4 represents a pulse–chase autoradiogram taken from one experiment, the multitude of conjugates of varying molecular weights, resulted in a smeared pattern, which is characteristic of the heterogeneous distribution of HMW-multiubiquitinated proteins. These HMW conjugates formed during the pulse were selectively degraded during the chase (Fig. 3). However, several other proteins that were conjugated to Ub during the pulse, ranging in molecular sizes between 20 kDa and 66 kDa, resisted the degradation during the chase.

To determine whether circadian rhythm has an effect on the Ub system, we kept a group of rats in the dark until 1000 (10D); the other group was left in the light until 2200 (22D; Figs. 3, 4). Although numerous conjugates were seen at 1000 (during the light cycle), the level of conjugates that was sustained when the animals continued in the dark for 3 more hours suggests that alterations in the levels of Ub conjugates are affected by circadian rhythm (Fig. 3B). However, after 3 additional hours in light, the level of conjugates dropped, with a wider range of standard deviation (25.7 ± 11.4%).

Multiple statistical comparisons showed that the differences in the mean values of the levels of Ub conjugates measured at 0700 were significant when compared with the levels at 1000 and 10D (P < 0.05). However, the differences in the levels of Ub conjugates measured at 1900 and 2200 were not significant. The data in Figure 3B indicate that Ub-conjugating activity was highest at 0700 (light on) and then declined significantly during the day (1000 and 1900) (P < 0.05). A slight increase in the activity was also seen at 2200. However, in the case of the metabolic activities of the conjugates, the values varied with the light–dark cycle (Fig. 3C). A higher loss of the conjugates was seen at 1000 and 1900 than at 0700 and 2200.

Immunocytochemical Localization of Light-Induced Retinal Ubiquitin Conjugates

There was a strong positive immunoreactivity of Ub and its conjugates in a representative retinal section of a light-exposed rat (Fig. 5A). Only a faint immunoreactivity was uniformly distributed throughout all the retinal cell layers from dark-adapted rats (Fig. 5B). The strong immunoreactivity in light-exposed retinas was localized at the border between the outer plexiform layer (OPL) (Fig. 5A, filled arrows) and inner nuclear layer (INL), and in the ganglion cell layer (GCL) (Fig. 5A, open arrows). These strongly immunoreactive cells at the junction of the inner nuclear
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A. Light Exposure Induces Retinal Ubiquitin Conjugation and Degradation 2351

A. Dark

B. Light

C. Light Dark

FIGURE 3. Effect of cyclic light and circadian rhythm on the conjugation and metabolism of the conjugates. Quantitation of the incorporation of $^{125}$I-Ubiquitin into retinal proteins and degradation and disassembly of the conjugates from four independent experiments were measured from retinal samples obtained from animals during their daily cyclic light treatment. The radioactivity in the conjugated forms of $^{125}$I-Ubiquitin shown in Figure 4 was quantitated by cutting the respective bands from each lane and counting in a $\gamma$-counter. (A) Light treatment. (B) Ubiquitin-conjugation reaction in 15-minute pulse at 37°C. Values were calculated as the percentage of $^{125}$I-Ubiquitin incorporated into retinal proteins in 15 minutes of incubation. (C) Degradation and disassembly of the conjugates after 30-minute chase. Values were calculated as the percentage of the conjugates degraded or disassembled in 30 minutes of incubation.

layer are interneuronal cells that form extensive lateral contacts within the plexiform layers to modulate the flow of the light signal. Higher magnification of the inner part of the outer plexiform layer of light-exposed retinas showed a densely stained network of thick processes (data not shown). From their morphologic appearance, these processes were identified as dendrites of horizontal cells. Results of preliminary electron microscopic studies showed that Ub immunoreactivity in retinal sections obtained from light-treated rats was uniformly distributed in the cytosol of the horizontal cells and their dendrites (data not shown). In the ganglion cell layer of the light-exposed retinas, immunoreactivity was constantly observed in the cell body of a large number of retinal neurons. The sections from the dark-adapted retinas showed no significant differences in the pattern of immunoreactivity between retinal cells.

Immunostaining was completely abolished when Ub antibody was adsorbed in vitro with 10 $\mu$M of free Ub or Ub-$\gamma$-globulin for 1 hour at room temperature before it was applied to the sections. Adsorption with $\gamma$-globulin did not suppress immunostaining. Blockage of the immunoreactivity in the inner retina and specifically in horizontal and ganglion cells was observed in the sections from the light-exposed animals.

The location of the light-induced Ub conjugates to horizontal and ganglion cells is very intriguing, in that these cells show strong immunoreactivity with an antibody raised against protein gene product 9.5.54 Protein gene product 9.5 is a neuron-specific protein, which has been regarded as a tissue-specific isozyme of Ub-carboxyl-terminal hydrolase.55 Carboxyl-terminal hydrolase participates in disassembling Ub conjugates formed during normal cell metabolism.55 It is interesting to study the correlation between the level of carboxyl-terminal hydrolase and the light-dependent increase in the level of Ub immunostaining in horizontal and ganglion cells. Similar to our finding with light stress, Müller cells, which are the major type of non-neuronal cells in the vertebrate retina, accumulate glial fibrillary acidic protein in response to neuronal injury56 and degeneration.57,58 Normally, these cells do

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not express glial fibrillary acidic protein. In support of our immunocytochemical data, it has been shown that Ub-protein conjugates also increased after transient ischemia in the hippocampus, a region in the brain known to be vulnerable to ischemia and hypoxia. More precise knowledge of the subcellular location of free and conjugated forms of Ub in light-exposed retinas would be helpful in understanding the functional significance of their presence in the visual system.

On the basis of the increase in Ub conjugation and degradation activities with bright light exposure and the established role of Ub in the cellular stress response, we propose that Ub is involved in protecting retinal cells from the stress that occurs during bright light exposure. The retina is extremely susceptible to peroxidation because of a combination of high oxygen flux and the presence of high levels of polyunsatu-
rated fatty acids in the rod outer segment membranes.\textsuperscript{22} It is also possible that bright light exposure may affect some retinal proteins through photodenu- 
turation or oxidation, which makes them more susceptible to Ub conjugation. Under this condition, Ub may contribute to retinal cells’ ability to withstand the stress caused by excess light exposure. If damaged proteins are not removed and destroyed, they may aggregate and contribute to the causes of cell death. Damaged proteins, aggregated proteins, and Ub conjugates frequently are the preferred substrates for the Ub proteolytic system.\textsuperscript{24}

In summary, our results are consistent with the hypothesis that Ub is a stress- and heat-shock protein. Although we clearly demonstrate the light activation of the Ub pathway, it will be of great interest to study the effect of Ub on specific retinal proteins in normal eyes and in animal models for different types of retinal disorders.

**Key Words**
diurnal rhythm, light exposure, retina, ubiquitin

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