Differential Effects of Rapamycin on Rods and Cones During Light-Induced Stress in Albino Mice

Kannan Kunchithapautham,¹ Beth Coughlin,¹ John J. Lemasters,² and Bärbel Rohrer^{1,3}

PURPOSE. Autophagy is a lysosomal machinery-dependent process that catabolizes cellular components/organelles and proteins in an autophagic vacuole (AV)-dependent and -independent manner, respectively. Short-term exposure of the retina to bright light results in shortening of the outer segments, concomitant with AV formation. Autophagy is also induced by continuous long-term light damage, leading to photoreceptor cell death. Here the authors examined two questions: is autophagy induced during light damage proapoptotic or antiapoptotic, and are rods and cones affected differently? To this end, Balb/c mice exposed to light damage were treated with rapamycin to increase autophagy.

METHODS. Balb/c and GFP-LC3 mice were treated with rapamycin/vehicle. Photoreceptor degeneration was induced by 10-day light damage. Autophagy was documented by histologic, biochemical, and molecular tools; rod and cone survival was assessed by histology and electroretinography.

RESULTS. Light damage resulted in rod, but not cone, cell loss. Autophagy and AV formation was elicited in response to light damage, which was amplified by rapamycin. Rapamycin treatment significantly improved rod survival and function, reduced apoptosis, and normalized cytokine production that was increased in light damage. However, AV formation in GFP-LC3 mice revealed that light damage or rapamycin treatment induced AVs in cones, concomitant with reduced cone-mediated electroretinograms.

CONCLUSIONS. Systemic rapamycin treatment provided rod protection; however, AV formation was induced only in cones. Thus, rapamycin may act differentially in stressed photoreceptors; rapamycin might protect rods by normalizing cytokine production, removing damaged proteins by AV-independent autophagy, or both, whereas cones might be protected by AV-dependent autophagy, possibly involving reduced photon capture. (*Invest Ophthalmol Vis Sci.* 2011;52:2967–2975) DOI: 10.1167/iovs.10-6278

From the ¹Department of Neurosciences, Division of Research and the Departments of ²Pharmaceutical Sciences and ³Ophthalmology, Medical University of South Carolina, Charleston, South Carolina.

Supported in part by National Institutes of Health Grants EY013520 (BR), DK37034 (JJL), and DK073336 (JJL); Hope for Vision (BR); and an unrestricted grant to the Medical University of South Carolina from Research to Prevent Blindness. BR is a Research to Prevent Blindness Olga Keith Weiss Scholar. Animal studies were conducted in a facility constructed with support from National Institutes of Health Grant C06 RR015455.

Submitted for publication July 27, 2010; revised September 30, 2010; accepted December 17, 2010.

Disclosure: **K. Kunchithapautham**, None; **B. Coughlin**, None; **J.J. Lemasters**, None; **B. Rohrer**, None

Corresponding author: Bärbel Rohrer, Department of Ophthalmology, Medical University of South Carolina, 167 Ashley Avenue, Charleston, SC 29425; rohrer@musc.edu.

Photoreceptors are the primary neurons in the retina responsible for the absorption of photons of light and the conversion of this chemical reaction to an electrical response. This specialization (i.e., the absorption of light and the energy requirements in the form of ATP and oxygen) generates an environment prone to oxidative damage.^{1,2} The photoreceptor membranes are rich in polyunsaturated fatty acids (PUFAs) that confer their fluidity and selective permeability. This high concentration of PUFAs makes these membranes especially vulnerable to oxidation. In addition, photoreceptors obtain all their oxygen from the vasculature of the choroid; however, the choroid cannot autoregulate blood flow. Thus, during times of low oxygen consumption (in the presence of light), photoreceptors experience a significant increase in oxygen tension (see Ref. 3 for review). Hence, excess light generates oxidative stress (see Ref. 4 for a summary), resulting in the generation of oxidized lipids⁵ and proteins⁶ and damaged mitochondria.⁷

However, the retina appears to be equipped with a number of mechanisms to protect its photoreceptors against light-induced or oxidative stress-mediated damage. Short bursts of light have been shown to increase antioxidants⁸ and neuroprotective factors⁹ in the retina, in addition to the circadian rhythm of basic fibroblast growth factor (bFGF) expression, which is increased during the day when photoreceptors are exposed to higher oxygen levels.³ Short-term exposure to bright light also triggers a mechanism of photostasis in which the outer segments containing the light-sensitive pigments are reduced in length to reduce photon capture.¹⁰ Reme et al.¹¹ have shown the presence of autophagic vacuoles (AVs) filled with rhodopsin-containing membranes in the rat photoreceptor inner segments during photostasis. Autophagy, which is a type II cell death, is the major pathway for the degradation of long-lived proteins and cytoplasmic organelles in animal cells. There are three main processes, referred to jointly as autophagy or individually as macroautophagy, microautophagy, and chaperone-mediated autophagy (CAM). Macroautophagy involves processes by which a cell compartmentalizes subcellular membranes and delivers them to the lysosomes, where they are degraded and recycled.^{12,13} Microautophagy involves the direct uptake of components by lysosomes.¹⁴ CAM involves selective uptake of certain proteins directly into the lysosomes.^{14,15} Macroautophagy is the only process that is characterized by the production of AVs.

In previous experiments analyzing molecular and biochemical events during long-term exposure to bright light, which leads to photoreceptor degeneration, we have shown an increase in lysosomal enzyme activity¹⁶ concomitant with the formation of AVs containing damaged organelles and cellular debris in the photoreceptor inner segments.⁷ Thus, under conditions of light stress, autophagy might contribute to photoreceptor stability by removing damaged proteins and subcellular membranes.

Autophagy can be stimulated by a number of conditions, including changes in environmental conditions such as nutrient deprivation, various hormonal stimuli, and other factors. A

convenient way to experimentally increase autophagy is to treat cells or organisms with rapamycin. Rapamycin is a lipophilic macrolide antibiotic and a negative regulator or mammalian target of rapamycin (mTOR) that has been shown to induce autophagy in many species ranging from yeast to mammals.¹⁷

Here, the hypothesis that autophagy might play a protective rather than a cell death-related role in light damage was tested. In addition, we investigated whether rods and cones might respond differently to rapamycin treatment. Balb/c mice exposed to 10 days of constant light (which eliminates $\geq 50\%$ of all the rod photoreceptors but does not affect cone survival) were analyzed. Rapamycin was administered daily during the damage period. Treated animals had significantly better rod photoreceptor survival and function than their control littermates after the 10 days of light damage, but cone function was reduced. Unexpectedly, rapamycin treatment was found to increase the generation of AVs in cones rather than rods in mice exposed to damaging light. The results are discussed in light of rapamycin as a multiple-target drug and the different processes of autophagy.

MATERIALS AND METHODS

Animals

Balb/c mice were generated from breeding pairs obtained from Harlan Laboratories (Indianapolis, IN). LC3-GFP was kindly provided by Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) and was genotyped by PCR according to published protocols.¹⁸ Because the LC3-GFP mice are on a C57BL/6 background and, therefore, resistant to light damage, an F1 cross between LC3-GFP and Balb/c mice was generated to sensitize the mice for light damage.¹⁹ Mice were housed in the Medical University of South Carolina animal care facility under a 12-hour light/12-hour dark cycle with access to food and water ad libitum. The ambient light intensity at the eye level of the mice was 85 ± 18 lux. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee. Light exposure experiments were performed on 3-month-old Balb/c mice. Light exposure consisted of constant fluorescent illumination of approximately 150 to 175 ft-c for 10 to 21 days, as described previously,¹⁶ ensuring that all cages were equidistant to the light source. Littermates were kept under the normal cyclic light conditions as controls.

Rapamycin Treatment

Rapamycin (LC Laboratories, Woburn, MA) was dissolved in 4% ethanol, 5.2% PEG400, and 5.2% Tween 80 and was given by intraperitoneal (IP) injection (10 mg/kg) once daily for 10 days. Body weight was monitored daily and was found not to change with rapamycin treatment (data not shown). Mice were killed 24 hours after the last injection of rapamycin.

Quantitative Real-Time PCR

Real-time PCR was performed as published previously.¹⁶ Total RNA (2 μ g each) was used to generate first-strand cDNA in reverse-transcription reactions (Invitrogen, Carlsbad, CA). PCR amplifications were conducted with a SYBR green PCR kit (QuantiTect; Qiagen, Valencia, CA) with 0.01 U/ μ L UNG enzyme (AmpErase; Applied Biosystems, Foster City, CA) to prevent carryover contamination. Real-time PCR was performed in triplicate in a sequence detection system (GeneAmp 5700; Applied Biosystems) using the following cycling conditions: 50°C for 2 minutes, 94°C for 15 minutes, 40 cycles of 94°C for 15 seconds, and 58°C for 1 minute. Quantitative values were obtained using the cycle number (C_t value), which is inversely proportional to the amount of a specific mRNA species in the tissue sample. Relative

gene expression levels were calculated using the equation $y = (1 + AE)^{\Delta\Delta C_t}$, where AE is the amplification efficiency of the target gene (set at 1.0 for all calculations) and $\Delta\Delta C_t$ is the difference between the mean experimental and control ΔC_t values. The ΔC_t value is the difference between the C_t value for the experimental gene and the β -actin internal reference control gene. The following primer sets, which were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) software, ensuring that the two primers span an intron, were used for this analysis: β -actin—forward, 5'-GCTACAGCTTCACCACCACA-3'; reverse, 5'-TCTCCAGGGAGGAAGAGGAT-3'; LC3—forward, 5'-CGGCTTCCTGTACATGGTTT-3'; reverse, 5'-ATGTGGGTGCCTACGTTCTC-3'; cathepsin S—forward, 5'-TCTAATCGGACATTGCTGACA-3'; reverse, 5'-CACAGCACTGAAAGCCCAACA-3'; lysozyme—forward, 5'-CCAGTGTCACGAGGCATTTA-3'; reverse, 5'-TGATAACAGGCTCATCTGTCTCA-3'; LAMP-2—forward, 5'-CACCCCACTCCAACTCCAACT-3'; reverse, 5'-TTGTGGCAGGGTTGATGTTA-3'; VEGF—forward, 5'-CAGGCTGCTGTAACGATGAA-3'; reverse, 5'-GCATTACATCTGCTGTGCT-3'; TGF- β —forward, 5'-TGGCTTGCAGAGATTA-3'; reverse, 5'-CGTCAAAAGACAGCCACTCA-3'.

Western Blot Analysis

To detect the presence of LC3, equal amounts (20 μ g) of protein/tissue lysate in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β -mercaptoethanol, 0.1% bromophenol blue) were loaded on a 4% to 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were blotted on a polyvinylidene difluoride membrane (Bio-Rad; 0.45- μ m pore size). Nonspecific binding was blocked in 5% blocking buffer (5% nonfat dry milk, 150 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.05% Tween-20) for 1 hour, and membranes were incubated overnight at 4°C with the primary antibodies against LC3 (Abgent, San Diego, CA). After five washes (150 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.05% Tween-20), the membranes were incubated for 2 to 4 hours at room temperature with horseradish peroxidase-coupled secondary antibodies (1:5000; Vector Laboratories, Burlingame, CA). The blots were developed with chemiluminescence reagents (Immobilon; Millipore Corporation, Billerica, MA) visualized in an Alpha imager (Alpha Innotech Corporation, San Leandro, CA). As a loading control, blots were stripped and reprobed with an antibody against GAPDH (Stressgen, Ann Arbor, MI).

Electroretinography

Electroretinographic (ERG) recordings were performed as described.¹⁹ Animals were dark-adapted overnight and anesthetized with xylazine and ketamine (20 and 80 mg/kg, respectively), and each pupil was dilated with 1 drop each of phenylephrine HCl (2.5%) and atropine sulfate (1%). Body temperature was stabilized with a DC-powered heating pad and held at 37°C. A needle ground-electrode was placed in the tail, and a needle reference electrode was placed in the forehead. ERG responses were measured with contact lenses containing a gold ring electrode held in place by 1 drop of methylcellulose. Rod electroretinograms were recorded with a visual electro-diagnostic testing system (EPIC-2000; LKC Technologies, Inc., Gaithersburg, MD) with a strobe-flash stimulus (Grass Technologies, West Warwick, RI). Cones were recorded with a modified diagnostic system (UTAS Visual Diagnostic System; LKC Technologies) replacing the blue LEDs with UV LEDs (λ_{max} , 365 nm) in a Ganzfeld (BigShot; LKC Technologies). ERG responses were recorded at a gain of 2000 using a notch filter at 60 Hz, bandpass filtered between 0.1 and 1500 Hz, and digitized online at 1 kHz with 12-bit accuracy. Stimuli to isolate rod responses consisted of 10- μ s single white flashes at a fixed intensity (2.48 photopic $cd \cdot s/m^2$ at the dome's inner surface, as calibrated by the manufacturer) under scotopic conditions. Stimuli to isolate cone responses consisted of 10- μ s single UV, green, or white flashes at a fixed intensity under photopic conditions (UV, 3.45; green, 3.73; white, 2.50 photopic $cd \cdot s/m^2$ at the dome's inner surface, as calibrated by the manufacturer). Single-flash responses were averaged two to four times with an inter-stimulus interval ranging from 1 second to 2 minutes, depending on

the photoreceptor system and the light intensity used. For all ERG recordings, a-wave amplitudes were measured from baseline to the a-wave trough, and b-wave amplitudes were measured from the a-wave trough or baseline to the peak of the b-wave.

Anatomy

After the ERG recordings, the animals were humanely killed, and their eyes were removed. The dorsoventral orientation was maintained, using the superior rectus muscle and the optic nerve as landmarks. Eyes used for semithin sections and light microscopy were removed, postfixed in 4% paraformaldehyde and 2% glutaraldehyde, and bisected dorsal to ventral through the optic nerve. Each half was embedded in an epoxy resin (EPON and Araldite; Electron Microscopy Sciences, Hatfield, PA) mixture, and sections were cut at 1 μm through the horizontal meridian and stained with toluidine blue. Photoreceptor layers were counted in the central (superior and inferior, within 350 μm of the optic nerve head) and peripheral (superior and inferior, within 350 μm of the ciliary body) retina. Three measurements were made per field and were averaged to provide a single value for each area, and the four area values were averaged to give a value for the retina as described previously.²⁰

Enzymatic Assay

Caspase-3 activity was measured in control and light-damaged Balb/c retinas with or without rapamycin, according to the manufacturer's instructions (Enzo Life Sciences, Plymouth Meeting, PA). Retinas were isolated, placed into lysis buffer at 4°C, sonicated, and centrifuged at 20,000g for 5 minutes. The supernatant was assayed for both protein content and caspase-3 activity. Protein content was measured using the Bradford-Folins reagent method (Bio-Rad Laboratories). The caspase-3 assay uses the fluorophore cresyl violet coupled to the C-terminus of the optimal tetrapeptide recognition sequence for caspase-3 (DEVD). Samples were read in a fluorometer equipped with a 592 nm excitation and a 628 nm emission filter. Data were expressed as change in relative fluorescent units (RFU) per milligram of protein.

Detection of LC3-GFP and Immunohistochemistry

In brief, eyes were collected after the appropriate treatment periods and were immersion fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 2 hours at 4°C, after which the anterior chamber and the lens were removed. After 2 washings in Tris-buffered saline, eyes were cryoprotected in 30% sucrose and embedded in optimal cutting media to generate 14 μm cryosections. For the detection of LC3-GFP, sections were washed in PBS, mounted in aqueous mounting medium (PermaFluor; Laboratory Vision, Thermo Fisher Scientific, Waltham, MA), and photographed. For colabeling with a cone-specific marker (PNA lectin), slides were washed in PBS, blocked with 10% normal goat serum and 3% bovine serum albumin (in PBS containing 0.4% Triton X), and incubated overnight in blocking solution containing PNA-lectin-FITC (Molecular Probes; now Invitrogen, Carlsbad, CA). Sections were analyzed using both a light microscope equipped for fluorescence with a digital camera driven by corresponding software (Axioscope; Zeiss, Thornwood, NY) and a confocal microscope (Leica, Bannockburn, IL). Cone cell counts were performed per window (magnification 40 $\times$, 420 μm window) in matching areas of the retina from three or more retinas per condition.

Statistical Analysis

All experiments described were performed at least in triplicate. Data are presented as mean $\pm$ SEM. For multiple measures per animal, data were analyzed by repeated-measures ANOVA followed by Fisher's post hoc test ($P < 0.05$); for analysis of single differences, one-way ANOVA was followed by Student's *t*-test analysis, accepting a significance level of $P < 0.05$.

RESULTS

Rapamycin Effects on Rod and Cone Survival and Function during Light Damage

To test the hypothesis that autophagy in light-induced photoreceptor degeneration is protective, animals were intraperitoneally injected daily with 10 mg/kg during the light damage period. Rapamycin effects on autophagy were documented using molecular, biochemical, and histologic readouts. Four markers for autophagy were analyzed using quantitative RT-PCR: LC3, which is specific for macroautophagy; two lysosomal enzymes, cathepsin S and lysozyme; and the CMA substrate receptor LAMP-2. All four markers were found to be upregulated after 10 days of light damage compared with cyclic light controls (Fig. 1A). Rapamycin treatment during cyclic light exposure increased LC3, cathepsin S, and lysozyme, but not LAMP-2 levels (Fig. 1B). Finally, rapamycin treatment during light damage increased LC3 mRNA levels, but not cathepsin S, lysozyme, or LAMP-2 levels compared with the PBS-treated animals (Fig. 1C). AV formation requires the recruitment of LC3 in its lipidated form (LC3II) to the AV membranes²¹; 10 days of rapamycin treatment also resulted in small, but significant, increases in LC3II levels (Figs. 2A–C; $P = 0.04$). Taken together, though light damage increased all four markers of autophagy in the retinal extracts, only LC3 mRNA and LC3II levels were increased by rapamycin over the levels generated by light damage only. In other words, only macroautophagy, but not microautophagy or CMA, was elevated by rapamycin treatment.

We have shown previously that 10 days of constant light (~ 1500 lux) reduces rod photoreceptor numbers in 3-month-old Balb/c mice by $\geq 50\%$.¹⁶ In addition, it severely impairs rod photoreceptors and overall retinal function.^{19,22} Here, we analyzed rows of photoreceptors in four locations in the retina (superior peripheral, superior central, inferior central, and inferior peripheral). As previously shown, 10 days of light damage significantly reduced the number of rows of photoreceptors throughout the retina, ranging from 2.6 ± 0.63 rows in the inferior central retina to 4.2 ± 0.52 rows in the superior peripheral retina (average retinal count, 3.25 ± 0.25). In contrast, rapamycin-treated animals lost significantly fewer photoreceptors during the same time period. In the same retinal regions, the rapamycin-treated animals had 4.3 ± 0.41 rows of photoreceptors in the inferior central retina and 5.4 ± 0.27 in superior peripheral retina (average retina count, 4.69 ± 0.29 ; Fig. 3A). When analyzed for all four regions using repeated-measures ANOVA, a treatment-specific effect ($P < 0.01$), but no treatment by region-specific effect ($P > 0.05$), was found. Given that rapamycin increases cell survival, apoptosis should be reduced. Caspase-3 activity was measured in the control and rapamycin-treated retinas exposed to constant light for 2 or 10 days. As shown in Figure 4, caspase-3 activity was increased ~ 3 - and ~ 7 -fold after 2 and 10 days of light exposure, which was significantly reduced by rapamycin treatment to ~ 2 - and ~ 5 -fold, respectively. Photoreceptor function was analyzed by electroretinography to determine whether the increase in survival was correlated with an increase in photoreceptor cell function (Fig. 3B). In ERGs, the negative-going potential (a-wave) reflects the response of the photoreceptors, whereas the positive-going potential (b-wave) reflects the response of the bipolar cells, the first postsynaptic neurons of the photoreceptors.²³ We used three light intensities that would allow us to record a-waves (maximum flash, and 6 and 10 dB of attenuation). Across the three light intensities, a-wave amplitudes in rapamycin-treated animals were approximately 26% to 44% larger than in control animals, resulting in a significant treatment-specific difference (repeated-measures ANOVA across

FIGURE 1. Quantitative RT-PCR for microautophagy, macroautophagy, and chaperone-mediated autophagy. Quantitative real-time PCR analysis was performed in Balb/c mice after light damage (A), after rapamycin treatment in the presence of cyclic light (B), and in mice reared in constant light with rapamycin treatment (C), using cyclic light-reared Balb/c animals as the control. Fold-change analysis showed that light damage increased mRNA levels for all four markers. Rapamycin in the presence of cyclic light increased markers of microautophagy and macroautophagy, but not of CMA (LAMP-2). Finally, rapamycin in the presence of light damage only increased LC3 mRNA above the levels generated after 10 days of light damage, suggesting that light damage-induced levels for the other three genes are already at a maximum. Data were expressed as mean $\pm$ SEM ($n = 3$). LD, light damage.

the three light intensities; $P < 0.05$; Fig. 3B); however, b-wave amplitudes were unaffected ($P = 0.2$).

In comparison, 10 days of light did not decrease cone photoreceptor cell numbers. Cone cell numbers were counted in five separate PNA lectin-stained tissues per condition (PNA lectin stains the cone outer segment sheath). Control sections contain on average 20.4 ± 0.5 PNA lectin-positive cones, and sections from vehicle-injected, light-exposed animals contain 20.8 ± 0.4 cones (not significant

[n.s.]), and rapamycin-injected and light-exposed animals contain 20.6 ± 0.5 cones (n.s.). Cone function was analyzed by electroretinography using ultraviolet, green, and white light stimuli to determine both whether cone function is impaired by light damage and whether cone ERGs are susceptible to rapamycin treatment. For photopic cone ERGs, the b-wave amplitudes were measured as a reflection of the cone responses because of the small amplitude of the cone a-wave. We examined one light intensity per wavelength (0 db). Before light damage, cone b-wave amplitudes reached values of 105.4 ± 4.9 (UV), 20.6 ± 1.6 (green), and 85.5 ± 4.2 (white). Those values were reduced to 31.5 ± 2.2 (UV), 15.8 ± 2.6 (green), and 36.1 ± 3.9 (white) in vehicle-treated mice, but were further reduced to 23.6 ± 2.7 (UV), 8.4 ± 2.1 (green), and 25.3 ± 3.6 (white) in rapamycin-treated animals. Across the three wavelengths, a significant treatment-specific difference (repeated-measures ANOVA; $P = 0.05$) could be established (Fig. 5).

FIGURE 2. Elevated LC3II protein levels in light-damaged Balb/c mice with rapamycin treatment. The cytoplasmic form of LC3 is LC3I; the lysosome-associated form is the lipidated LC3II. (A) Western blot analysis was performed to quantify changes in LC3I and LC3II levels in response to light damage and rapamycin treatment. GAPDH was used as a loading control and to normalize LC3I and LC3II levels (B, C). As shown previously, light damage results in an increase in levels of LC3I and LC3II. Daily rapamycin injections during the light damage period significantly increased the levels of LC3II at 10 days, confirming the results seen at the mRNA level. Data were expressed as mean $\pm$ SEM ($n = 3$). LD, light damage.

FIGURE 3. Rapamycin treatment improved rod photoreceptor cell counts and rod photoreceptor cell function in retinas undergoing light damage. Balb/c mice (3 months of age) were injected daily with either PBS (control) or rapamycin during the 10-day light damage period. (A) Cell counts (i.e., rows of photoreceptors in a horizontal Epon section) revealed that rapamycin-treated animals had significantly more photoreceptors remaining. Plotted is the average count across the four regions: dorsal peripheral, dorsal central, ventral central, and ventral peripheral. (B) ERG recordings at three different light intensities (unattenuated light, and 0.6 and 1 log unit of attenuation) showed that rapamycin significantly improved rod photoreceptor function as determined by a-wave analysis ($P < 0.01$). (C) B-wave amplitudes recorded over the same light-intensity range were unaffected ($P = 0.2$).

Taken together, the results thus far have shown that the induction of autophagy by rapamycin treatment is correlated with increased rod photoreceptor cell survival and function but decreased cone-mediated retinal function.

Rapamycin Effects on AV Formation

In a previous publication on the light-damaged albino mouse, we demonstrated the presence of AVs by electron microscopy⁷; however, no attempt was made to determine whether those AVs were present in rods or cones. Here we examined the occurrence of AVs after light damage or rapamycin treatment in the retina by light microscopy using GFP-LC3 trans-

genic mice.¹⁸ These mice were generated by fusing the rat LC3, the mammalian homolog of atg8, to the C terminus of EGFP and insertion behind the ubiquitous CAG promoter. The selected line not only has ubiquitous GFP-LC3 expression, but the transgene is thought to have integrated into a pseudogene locus and is unlikely to have any deleterious effects.²⁴ To obtain GFP-LC3 mice that are sensitive to constant light damage, an F1 generation between GFP-LC3 (on C57BL/6) and Balb/c mice was generated. In previous experiments,¹⁹ one copy of the Leu450 Rpe65 allele required for sensitivity to constant light damage²⁵ was found to be sufficient. GFP-LC3 is expressed throughout the retina, and, like its untagged counterpart, LC3, is found exclusively in the cytoplasm (Fig. 6A). After exposure to constant light for either 10 or 21 days (Figs. 6C, 6E), but not for 4 days (Fig. 6B), bright GFP-LC3 punctae could be visualized in both the synaptic pedicles and the inner segments. AVs have previously been shown by us in these two locations by electron microscopy.⁷ Rapamycin injections ac-

FIGURE 4. Rapamycin reduced the levels of caspase-3 activity in retinas undergoing light damage. Caspase-3 assay was performed on the retinal extracts from control and 10-day light damaged Balb/c mice with or without rapamycin treatment. Enzyme activity was expressed as RFU in percent per milligram protein per each sample. Caspase-3 activity levels were increased by ~6.5-fold after 10 days of light damage; rapamycin significantly blunted this increase. Data were expressed as mean $\pm$ SEM ($n = 3$). LD, light damage.

FIGURE 5. Rapamycin treatment reduced cone photoreceptor cell function in retinas undergoing light damage. Balb/c mice (3 months of age) were injected with either PBS (control) or rapamycin daily during the 10-day light damage period. ERG recordings at three different wavelengths (white, green, and UV) showed that rapamycin significantly reduced cone photoreceptor function as determined by b-wave analysis ($P < 0.05$).

FIGURE 6. GFP-LC3 marks autophagosomes in mouse photoreceptors. GFP-LC3 mice (C57BL/6) were crossed with Balb/c to obtain mice sensitive to light damage. GFP fluorescence was analyzed using confocal microscopy in radial sections. (A–F) Images were obtained using a 40× oil immersion lens; (a–f) are magnified portions of each panel, respectively. In control mice (A, a), GFP-LC3 was diffusely distributed. After 4 days of light damage (B, b), GFP-LC3 fluorescence increased, but no distinct punctae of autophagosomes could be distinguished. Autophagosomes could be identified in the inner segments and synaptic pedicles at 10 days of light damage (C, c). Interestingly, in animals treated with rapamycin, autophagosome formation was accelerated, allowing visualization by 4 days of light damage (D, d), a process that was even more pronounced by 10 days (E, e). Rapamycin treatment alone for 10 days also induced autophagosome induction (F, f). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; LD, light damage; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments.

celerated AV formation such that 4 days of light damage were found to be sufficient to generate the bright GFP-LC3 punctae normally only seen after 10 days of light damage (Fig. 6D); rapamycin injections also resulted in AV formation independent of light damage stress (Fig. 6F). Interestingly, the localization of GFP-LC3 appears to be in cones. When sections were colabeled with PNA lectin, all GFP-LC3 punctae were aligned with PNA lectin-positive cone sheaths (Fig. 7).

Hence, the histology suggests that the induction of AVs by rapamycin treatment is independent of rod photoreceptor cell survival and function, but may be responsible for the decrease in cone-mediated retinal function.

Rapamycin as a Multi-Target Molecule

Rapamycin increases rod survival, but the effect might not be attributable to AV-dependent autophagy. Rapamycin is a multi-target molecule that activates autophagy-independent pathways, which include the inhibition of VEGF activity,²⁶ wound healing,²⁷ general immunosuppression,^{27,28} and inhibition of translation.^{29,30} Bemelmans and coworkers (Bemelmans A et al. *IOVS*. 2007;48:ARVO E-Abstract 1692) have shown that lentiviral transfer of anti-VEGF antibody to the mouse retina protected against light-induced photoreceptor degeneration. In addition, VEGF has been shown to be proapoptotic in the presence of increased TGF- β .³¹ Here we examined VEGF and TGF- β mRNA levels in retinas exposed to 10 days of light damage in the presence and absence of rapamycin treatment (Fig. 8). We confirmed that VEGF expression is increased in light damaged retinas by almost 3-fold (2.9 ± 0.3), and showed for the first time that TGF- β is significantly elevated approximately 4.5-fold (4.5 ± 0.1). Rapamycin normalized VEGF levels (1.0 ± 0.1) and significantly blunted the increase in TGF- β (2.1 ± 0.1).

In summary; the molecular studies suggest that rapamycin treatment may result in increased rod photoreceptor cell sur-

vival and function not by activating macroautophagy, but by normalizing VEGF and TGF- β levels.

DISCUSSION

The main findings of this study are as follows: daily rapamycin treatment improved rod photoreceptor cell survival and function in mice undergoing light stress, but reduced cone photoreceptor-mediated retinal function; GFP-LC3 is a powerful method with which to monitor AV formation in mouse photoreceptors; rapamycin increases the generation of AVs in cone photoreceptors under light stress conditions; and rapamycin effects on rod photoreceptor cell survival may be attributed to its normalizing effect on VEGF and TGF- β levels.

Photoreceptor degeneration from light stress in the mouse has shown that caspase-dependent and caspase-independent cell death mechanisms, such as the activation of cysteine proteases, lysosomal proteases, autophagy, and complement-mediated lysis, appear to be involved in this process.¹⁶ However, the hypothesis that autophagy might play a protective rather than cell death-related role in light damage has not yet been tested. Autophagy is typically recognized in the context of cell death. Three different types of cell death have been distinguished: apoptosis, autophagy, and necrosis. Autophagy, or type 2 cell death, is the major pathway for the degradation of long-lived proteins and cytoplasmic organelles in animal cells and, thus, involves processes by which a cell compartmentalizes subcellular membranes and delivers them to the lysosomes, where they are degraded and recycled.^{12,13} However, autophagy not only removes damaged cellular components, it can also be stimulated by a change of environmental conditions such as nutrient deprivation, various hormonal stimuli, and other factors. As such, autophagy plays an important role in both normal development and tissue remodeling and in pathologic conditions, but autophagy also promotes cell survival

FIGURE 7. GFP-LC3 marks autophagosomes in cone photoreceptors. To confirm the cellular localization of autophagosomes in GFP-LC3 mice, sections were counterstained with Texas Red-tagged peanut agglutinin (PNA) lectin to identify cones. Texas Red and GFP fluorescence were analyzed using confocal microscopy in radial sections. No light damage mice were used for control staining (A–C). LC3-GFP-positive inner segments were shown to align with PNA lectin-positive cone outer segments (D–F). *Insets:* higher magnification images of the cones. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments.

under certain circumstances by recycling amino acids and other cellular components to maintain proper cell function. Autophagy is involved in eliminating protein aggregates that often appear in a variety of neurodegenerative diseases^{32–34}, while cancer cells rely on autophagy for survival.³⁵ Autophagy contributes to cell damage and degrades damaged organelles such as mitochondria,³⁶ as well as intracellular bacteria and viruses.^{37,38} Additional examples relevant to the experiment described here show that autophagy may act as a central regulatory mechanism of cell growth and axonal remodeling. Komatsu et al.³⁹ have shown that the induction of autophagy serves as an early stress response in axonal dystrophy and may participate in the remodeling of axon structures.³⁹ Vellai et al.⁴⁰ have reported that insulin/insulin-like growth factor receptor-1 and TGF- β act as major growth regulatory pathways converging on autophagy genes to control cell size. Thus, overall, autophagy plays an important role during normal cellular remodeling and has cytoprotective functions during metabolic stress, aging, and intracellular infection, and in certain neurodegenerative diseases.

Rapamycin, as mentioned in the Introduction, is a negative regulator, or mTOR, that has been used in many species to induce autophagy.¹⁷ However, despite its wide use, the mechanism by which mTOR regulates mammalian autophagy is still unclear. Several studies have shown that rapamycin, acting through the mTOR pathway, is neuroprotective in various neurologic diseases. The induction of autophagy by rapamycin has been shown to increase the clearance of cytoplasmic, aggregate-prone proteins and to reduce the number of such aggregates.⁴¹ Thus, the amelioration of neurodegenerative symptoms by autophagic induction has been seen in Huntington disease models⁴² and in models of Parkinson disease⁴³ and Alzheimer disease.⁴⁴ Autophagy is also a final cell protection strategy deployed against endoplasmic reticulum-accumulated

cytotoxic aggregates that cannot be removed by the normal endoplasmic reticulum-associated degradation pathway.⁴⁵ Overall, the neuroprotective effects of rapamycin are thought to be partially mediated by enhanced degradation of misfolded proteins by autophagy. However, additional mechanisms, such as the increase in nutrients provided by autophagy or the removal of oxidized proteins and lipids as well as damaged organelles, could contribute to the overall effect. Recently, rapamycin was found to increase longevity in mice, possibly acting through mTOR targets.⁴⁶ Finally, when interpreting the effects of rapamycin, it is important to consider that not only does mTOR regulate a range of pathways, rapamycin itself is not a single-target drug. The major side effects of rapamycin, however, can be attributed primarily to autophagy-independent pathways, which include the inhibition of VEGF activity,²⁶ wound healing²⁷ and general immunosuppression.^{27,28} In addition, mTOR increases protein synthesis, or, on the flip side, rapamycin decreases translation by inhibiting the ribosomal S6 kinases, reducing eukaryotic translation initiation factor 4E (4E/BP)- or eukaryotic elongation factor eEF2-mediated translation.^{29,30}

We and others have studied autophagy in the retina, in particular in the context of photoreceptor remodeling and degeneration. Reme et al.¹¹ have shown that AVs are generated during short-term exposure to bright light and have speculated that they might be involved in photostasis, a mechanism to shorten photoreceptor outer segments to reduce photon capture.¹⁰ Similarly, we have shown that long-term exposure to bright light results in the formation of AV, containing damaged mitochondria and cellular debris in the inner segments, and damaged synaptic vesicles in the photoreceptor synapses; however, we did not aim to identify whether these AVs were associated with rods or cones.⁷ Our original work focused predominantly on identifying the possible interaction between autophagy, apoptosis, and necrosis. Using 661W photoreceptor cells challenged with H₂O₂ as a model for light-induced (i.e., oxidative stress-induced) photoreceptor degeneration, we showed that blocking autophagy significantly decreased caspase-3 activity, whereas blocking apoptosis increased the formation of AVs. Either treatment alone slowed 661W cell degeneration, whereas adding the two inhibitors simultaneously resulted in increased necrosis. Together, that data suggest that in photoreceptor cell death, autophagy may play a role in apoptosis by either delaying it or by preceding and eventually initiating it.⁷

FIGURE 8. Quantitative RT-PCR for VEGF and TGF- β . Quantitative real-time PCR analysis was performed in Balb/c mice after light damage in the presence and absence of rapamycin treatment using cyclic light-reared Balb/c animals as the control. Fold-change analysis showed that PBS-injected animals (control) have increased levels of VEGF and TGF- β mRNA compared with cyclic light-reared mice; rapamycin was found to reduce these levels back to baseline (VEGF) or to blunt it significantly. Data were expressed as mean $\pm$ SEM ($n = 3$).

Here, we confirmed that in the intact retina, light stress activated the lysosomal-mediated autophagic pathway and triggered the formation of AVs as measured by the increased levels of lysozyme, cathepsin S, and LAMP2 (Fig. 1), the increased expression of LC3 mRNA (Fig. 1), and the lipidated forms of LC3 and LC3II (Fig. 2), respectively. However, based on these readouts, only macroautophagy, but not microautophagy and CMA, could be augmented by rapamycin treatment. To test whether, under light stress conditions, rapamycin treatment might contribute to photoreceptor stability, photoreceptor survival and function were tested after 10 days of light damage in the presence or absence of daily injections of rapamycin. Rapamycin was found to improve rod photoreceptor survival and function (Figs. 3A, 3B) compared with vehicle-treated animals; yet rod output, as determined by b-wave analysis, was unaffected (Fig. 3C). On the other hand, though cone survival was unaffected by either light damage or rapamycin treatment, animals treated with rapamycin had significantly reduced cone photoreceptor cell-mediated retinal responses, when tested with UV, green, and white light test flashes (Fig. 5). Image analysis revealed that GFP-LC3-positive AVs are localized to cones, but not rods (Figs. 6, 7). Thus, the effects of rapamycin on rods appear to be macroautophagy independent, whereas in cones, macroautophagy might play an essential role in altering the cone-mediated ERG response. Cone b-wave amplitudes might be reduced by autophagy, participating in the shortening of the cone outer segments and by removing the light-sensitive pigment (photostasis). Alternatively, autophagy-dependent or -independent changes in signaling at the cone-bipolar cell synapse might result in reduced cone b-wave amplitudes. Similar autophagy-independent pathways might be responsible for the lack of an improvement in rod b-wave amplitudes in light of the increased rod responses. On a separate note, the differential effects on structure and function (i.e., improved or no effect on rod and cone survival vs. the lack of an effect or a negative effect on rod and cone b-wave amplitudes, respectively) highlights the importance of investigating both aspects of a cell when testing therapeutic compounds. It has been suggested that rapamycin, acting through the mTOR pathway, prevents or reduces neurodegeneration in a number of neurologic diseases. Thus far, inhibition or activation of the mTOR pathway has been used in two different models of retinal degeneration. Kaushal⁴⁷ has shown that P23H rhodopsin, a misfolding mutant that is retained in the endoplasmic reticulum, can be successfully removed by increasing autophagy in a cellular expression system, suggesting that this mechanism might be useful in clearing these misfolded proteins associated with retinal degeneration *in vivo*. On the other hand, the induction of the insulin/mTOR pathway and, hence, the reduction of autophagy was found to delay cone cell death in a mouse model of retinitis pigmentosa, presumably by reducing starvation in the remaining cones.⁴⁸ Here, in light damage, the mTOR pathway might be involved in increasing AVs in cones, thus aiding in the removal of damaged cellular components. It is unclear why AVs could not be documented in rods, as previously shown by Reme et al.¹¹ in the rat, but the difference might include the experimental paradigm (a light intensity switch during the day vs. constant light) and the timing (1–3 days after the light stimulus onset vs. 10 days). Rapamycin has been shown in muscle cells to cause a metabolic shift from glucose use to fatty acid oxidation,⁴⁹ reducing glycolysis by approximately 40%. Although we found that light damage reduced the expression of 6-phosphofructokinase, the rate-limiting protein for glycolysis,¹⁶ rapamycin did not further affect 6-PFK mRNA levels (data not shown), making it unlikely that the rapamycin effects occurred through changes in metabolism, although this possibility cannot be excluded. Finally, rapamycin is not a single-target molecule. It has been shown

that rapamycin activates a number of autophagy-independent pathways. Rapamycin has been used to inhibit VEGF expression and activity,²⁶ to affect wound healing,²⁷ to act as a general immunosuppressant,^{27,28} and to decrease protein synthesis by inhibiting translation.^{29,30} As mentioned above, blocking VEGF activity has been shown to provide neuroprotection in light damage (Bemelmans A et al. *IOVS*. 2007; 48: ARVO E-Abstract 1692), and VEGF has been shown to be proapoptotic in the presence of increased TGF- β .³¹ Increased levels of VEGF and TGF- β in light damage that could be eliminated or blunted by rapamycin suggest that the effect of rapamycin on rod survival is dependent on this autophagy-independent pathway. Finally, because rapamycin is administered systemically, protective effects produced by autophagy-independent mechanisms on other cells in the eye, or even elsewhere in the body, cannot be excluded.

In summary, in this study, we have demonstrated that rapamycin increases rod photoreceptor cell survival and function in the light damage model, but that it reduces cone-mediated retinal function. Macroautophagy was found to be activated during light stress in photoreceptor inner segments and synaptic pedicles in cones only, not in rods. Increased rod photoreceptor protection by rapamycin was found to be correlated with reduced caspase activity and normalized levels of proapoptotic cytokines. Thus, rapamycin has differential effects on rods and cones during light stress, both of which might contribute independently to photoreceptor cell survival.

Acknowledgments

The authors thank Luanna Bartholomew for critical review.

References

- Ames A 3rd, Li YY, Heher EC, Kimble CR. Energy metabolism of rabbit retina as related to function: high cost of Na⁺ transport. *J Neurosci*. 1992;12:840–853.
- Demontis GC, Longoni B, Gargini C, Cervetto L. The energetic cost of photoreception in retinal rods of mammals. *Arch Ital Biol*. 1997;135:95–109.
- Stone J, Maslim J, Valter-Kocsi K, et al. Mechanisms of photoreceptor death and survival in mammalian retina. *Prog Retin Eye Res*. 1999;18:689–735.
- Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res*. 2005;24:275–306.
- Dentchev T, Yao Y, Pratico D, Dunaief J. Isoprostane F2 α -VI, a new marker of oxidative stress, increases following light damage to the mouse retina. *Mol Vis*. 2007;13:190–195.
- Tanito M, Masutani H, Nakamura H, Oka S, Ohira A, Yodoi J. Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. *Neurosci Lett*. 2002;326:142–146.
- Kunchithapautham K, Rohrer B. Apoptosis and autophagy in photoreceptors exposed to oxidative stress. *Autophagy*. 2007;3:433–441.
- Penn JS, Naash MI, Anderson RE. Effect of light history on retinal antioxidants and light damage susceptibility in the rat. *Exp Eye Res*. 1987;44:779–788.
- Liu C, Peng M, Laties AM, Wen R. Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J Neurosci*. 1998;18:1337–1344.
- Penn JS, Williams TP. Photostasis: regulation of daily photon-catch by rat retinas in response to various cyclic illuminances. *Exp Eye Res*. 1986;43:915–928.
- Reme CE, Wolftrum U, Imsand C, Hafezi F, Williams TP. Photoreceptor autophagy: effects of light history on number and opsin content of degradative vacuoles. *Invest Ophthalmol Vis Sci*. 1999;40:2398–2404.
- Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6:463–477.

13. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science*. 2000;290:1717-1721.
14. Todde V, Veenhuis M, van der Klei IJ. Autophagy: principles and significance in health and disease. *Biochim Biophys Acta*. 2009;1792:3-13.
15. Dice JF. Chaperone-mediated autophagy. *Autophagy*. 2007;3:295-299.
16. Lohr HR, Kuntchithapatham K, Sharma AK, Rohrer B. Multiple, parallel cellular suicide mechanisms participate in photoreceptor cell death. *Exp Eye Res*. 2006;83:380-389.
17. Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. *Autophagy*. 2008;4:851-865.
18. Mizushima N, Kuma A. Autophagosomes in GFP-LC3 transgenic mice. *Methods Mol Biol*. 2008;445:119-124.
19. Richards A, Emondi AA, Rohrer B. Long-term ERG analysis in the partially light-damaged mouse retina reveals regressive and compensatory changes. *Vis Neurosci*. 2006;23:91-97.
20. Rohrer B, Matthes MT, LaVail MM, Reichardt LF. Lack of p75 receptor does not protect photoreceptors from light-induced cell death. *Exp Eye Res*. 2003;76:125-129.
21. Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000;19:5720-5728.
22. Rohrer B, Guo Y, Kunchithapatham K, Gilkeson GS. Eliminating complement factor D reduces photoreceptor susceptibility to light-induced damage. *Invest Ophthalmol Vis Sci*. 2007;48:5282-5289.
23. Pugh ENJ, Falsini B, Lyubarsky AL. *The Origin of the Major Rod- and Cone-Driven Components of the Rodent Electroretinogram and the Effect of Age and Light-Rearing History on the Magnitude of These Components*. New York: Plenum Press; 1998:93-128.
24. Mizushima N. Methods for monitoring autophagy. *Int J Biochem Cell Biol*. 2004;36:2491-2502.
25. Danciger M, Lyon J, Worrell D, et al. New retinal light damage QTL in mice with the light-sensitive RPE65 LEU variant. *Mamm Genome*. 2004;15:277-283.
26. Gasperini P, Tosato G. Targeting the mammalian target of rapamycin to inhibit VEGF and cytokines for the treatment of primary effusion lymphoma. *Leukemia*. 2009;23:1867-1874.
27. Knight RJ, Villa M, Laskey R, et al. Risk factors for impaired wound healing in sirolimus-treated renal transplant recipients. *Clin Transplant*. 2007;21:460-465.
28. Di Benedetto F, Di Sandro S, De Ruvo N, et al. Immunosuppressive switch to sirolimus in renal dysfunction after liver transplantation. *Transplant Proc*. 2009;41:1297-1299.
29. Raught B, Gingras AC, Sonenberg N. The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci U S A*. 2001;98:7037-7044.
30. Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J*. 2001;20:4370-4379.
31. Ferrari G, Pintucci G, Seghezzi G, Hyman K, Galloway AC, Mignatti P. VEGF, a prosurvival factor, acts in concert with TGF-beta1 to induce endothelial cell apoptosis. *Proc Natl Acad Sci U S A*. 2006;103:17260-17265.
32. Cardoso SM, Pereira CF, Moreira PI, Arduino DM, Esteves AR, Oliveira CR. Mitochondrial control of autophagic lysosomal pathway in Alzheimer's disease. *Exp Neurol*. 2010;223:294-298.
33. Irrcher I, Park DS. Parkinson's disease: to live or die by autophagy. *Sci Signal*. 2009;2:pe21.
34. Rusten TE, Simonsen A. ESCRT functions in autophagy and associated disease. *Cell Cycle*. 2008;7:1166-1172.
35. Brech A, Ahlquist T, Lothe RA, Stenmark H. Autophagy in tumour suppression and promotion. *Mol Oncol*. 2009;3:366-375.
36. Tolkovsky AM, Xue L, Fletcher GC, Borutaite V. Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? *Biochimie (Paris)*. 2002;84:233-240.
37. Campoy E, Colombo MI. Autophagy in intracellular bacterial infection. *Biochim Biophys Acta*. 2009;1793:1465-1477.
38. Lee HK, Iwasaki A. Autophagy and antiviral immunity. *Curr Opin Immunol*. 2008;20:23-29.
39. Komatsu M, Wang QJ, Holstein GR, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci U S A*. 2007;104:14489-14494.
40. Vellai T, Bicsak B, Toth ML, Takacs-Vellai K, Kovacs AL. Regulation of cell growth by autophagy. *Autophagy*. 2008;4:507-509.
41. Ravikumar B, Berger Z, Vacher C, O'Kane CJ, Rubinsztein DC. Rapamycin pre-treatment protects against apoptosis. *Hum Mol Genet*. 2006;15:1209-1216.
42. Ravikumar B, Vacher C, Berger Z, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet*. 2004;36:585-595.
43. Pan T, Kondo S, Le W, Jankovic J. The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease. *Brain*. 2008;131:1969-1978.
44. Boland B, Kumar A, Lee S, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci*. 2008;28:6926-6937.
45. Ishida Y, Yamamoto A, Kitamura A, et al. Autophagic elimination of misfolded procollagen aggregates in the endoplasmic reticulum as a means of cell protection. *Mol Biol Cell*. 2009;20:2744-2754.
46. Harrison DE, Strong R, Sharp ZD, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*. 2009;460:392-395.
47. Kaushal S. Effect of rapamycin on the fate of P23H opsin associated with retinitis pigmentosa (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc*. 2006;104:517-529.
48. Punzo C, Kornacker K, Cepko CL. Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa. *Nat Neurosci*. 2009;12:44-52.
49. Sipula JJ, Brown NF, Perdomo G. Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation. *Metabolism*. 2006;55:1637-1644.