Loss of Neuroprotective Survival Signal in Mice Lacking Insulin Receptor Gene in Rod Photoreceptor Cells

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Insulin receptor (IR) signaling provides a trophic signal for transformed retinal neurons in culture, but the role of IR activity in vivo is unknown. We previously reported that light causes increased tyrosine phosphorylation of the IR in vivo, which leads to the downstream activation of the phosphoinositide 3-kinase and Akt pathway in rod photoreceptor cells. The functional role of IR in rod photoreceptor cells is not known. We observed that light stress induced tyrosine phosphorylation of the IR in rod photoreceptor cells, and we hypothesized that IR activation is neuroprotective. To determine whether IR has a neuroprotective role on rod photoreceptor cells, we used the Cre/lox system to specifically inactivate the IR gene in rod photoreceptors. Rod-specific IR knock-out mice have reduced the phosphoinositide 3-kinase and Akt survival signal in rod photoreceptors. The resultant mice exhibited no detectable phenotype when they were raised in dim cyclic light. However, reduced IR expression in rod photoreceptors significantly decreased retinal function and caused the loss of photoreceptors in mice exposed to bright light stress. These results indicate that reduced expression of IR in rod photoreceptor cells increases their susceptibility to light-induced photoreceptor degeneration. These data suggest that the IR pathway is important for photoreceptor survival and that activation of the IR may be an essential element of photoreceptor neuroprotection.

Insulin receptor (IR) signaling provides a trophic signal for transformed retinal neurons in culture (1), but the role of the IR in vivo is unknown. IR activation has been shown to rescue retinal neurons from apoptosis through a phosphoinositide 3-kinase (PI3K) cascade (1). We previously reported that light induces tyrosine phosphorylation of the retinal IR and that this activation leads to the binding of PI3K to rod outer segment (ROS) membranes (2). More recently, we demonstrated that IR activation is mediated through the G-protein-coupled receptor rhodopsin (3). IR signaling is also involved in 17β-estradiol-mediated neuroprotection in the retina (4). Recent evidence suggests a down-regulation of IR kinase activity in diabetic retinopathy that is associated with the deregulation of downstream signaling molecules (5). Deletion of several downstream effector molecules of the IR signaling pathway, such as IRS-2 (6), Akt2 (7), and Bcl-xl (8), in the retina resulted in a photoreceptor degeneration phenotype. These studies clearly indicate the importance of the IR signaling pathway in the retina.

The IR is highly conserved, and the high degree of IR homology between Caenorhabditis elegans, Drosophila, and humans suggests functional conservation in mammalian retina. The IR regulates neuronal survival in C. elegans (9). In Drosophila, the IR serves an important function to guide retinal photoreceptor axons from the retina to the brain during development (10), and the IR influences the size and number of photoreceptors (11). The lack of IR activation leads to neurodegeneration in brain/neuron-specific IR knock-out mice (12). Dysregulation of insulin signaling in the central nervous system has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer and Parkinson disease (13, 14). These studies clearly suggest that the IR pathway is important for neuronal survival and maintenance.

Mice lacking IRs are born with normal features but develop early postnatal diabetes and die of ketoacidosis (15, 16). Tissue-specific (Cre/lox) deletion of the IR in various tissues shows distinct phenotypes (17), including dyslipidemia in muscle (18), impaired glucose tolerance in adipose tissues (19), moderate insulin resistance and transient hyperglycemia in liver (20), impaired glucose tolerance in pancreatic β-cells (21), β-cell failure in brown adipocytes (22), obesity and reduced fertility in brain tissues (23), and protection against retinal neovascularization in vascular endothelial cells (24). The functional role of IRs in retina or photoreceptor cells is not known.

In this study we demonstrate that stress induced the activation of the IR in the retina, especially in rod photoreceptor cells. To determine the functional role of IR in rod photoreceptor cells, we generated a conditional IR knock-out mouse under the control of the rod opsin promoter by using Cre/lox technology.

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2 The abbreviations used are: IR, insulin receptor; PI3K, phosphoinositide 3-kinase; IRβ, IR β subunit; ROS, rod outer segments; PTP-1B, protein-tyrosine phosphatase-1B; ONL, outer nuclear layer; ERG, electroretinogram; PIPES, 1,4-piperazinediethanesulfonic acid; PI-4,5-P2, phosphatidylidyinositol 4,5-biphosphate; PI-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate.
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Reduced expression of IR led to reduced PI3K and Akt association with rod outer segment (ROS) membranes. Reduced expression of the IR in photoreceptor cells caused increased sensitivity to light-induced photoreceptor degeneration.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-IRβ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific polyclonal anti-IR/IGF-1R (Tyr(1158)/Tyr(1162)/Tyr(1163)) antibody was obtained from BIOSOURCE. Anti-p85, anti-α1-Na+K+-ATPase, and anti-PTP-1B antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Akt, anti-pAkt (Ser473), and anti-caspase-3 antibodies were obtained from Cell Signaling (Danvers, MA). Monoclonal anti-α1-antithrombin antibody was obtained from BIOSOURCE. Anti-p85, anti-IR/IGF-1R (Tyr(P)1158/Tyr(P)1162/Tyr(P)1163) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific antibodies were used to detect phosphorylation at specific residues of the IR.

Animals—All animal work was in strict accordance with the NIH Guide for the Care Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All protocols were approved by the IACUC at the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. Sprague-Dawley (Harlan Sera-Lab, Indianapolis, IN) rats were born and raised in our vivarium and kept under dim cyclic light (5 lux, 12 h on/off, 7 a.m. to 7 p.m.) prior to experimentation.

Generation of Photoreceptor-specific IR Knock-out Mice—The targeting vector was constructed using the mouse IR gene in which a selection cassette flanked by loxP sites was introduced upstream of exon 4 with a third loxP site downstream of exon 4 (21). In the presence of Cre recombinase, floxed exon 4 of the IR allele would be deleted, thereby causing a frameshift mutation and an immediate stop of translation. The predicted product of this gene, if one exists, would represent a 308-amino acid fragment. The IR floxed homozygous mice (backcross) were bred with the IRfloxed homozygous mice and at the periphery 100 mm from the inferior and superior edge of the retina by using Image J (1.32j) software (developed by Wayne Rasband, National Institutes of Health, Bethesda, available online, rsb.info.nih.gov).

Electroretinography—Flash ERGs were recorded by using a Ganzfeld-type ERG recording system (UTAS-E3000, LKG Technologies Inc., Gaithersburg, MD) as reported previously (28). All animals were dark-adapted for 16 h before being tested. After anesthesia was induced by intramuscular injection of a mixture of ketamine (120 mg/kg) and xylazine (6 mg/kg), the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceutical, Osaka, Japan). Gold electrodes were placed on both eyes. An identical reference electrode was placed in the mouth, and a ground electrode was placed on the tail. A single flashlamp (25 db for 10 ms) from a halogen source was used as the light stimu-
uls. The a-wave amplitude was measured as the difference in voltage between base line just before the flash and the peak of a-wave, and the b-wave amplitude was measured as difference in voltage between peaks of a- and b-waves. The a- and b-wave amplitudes were obtained from the right and left eyes were averaged for each animal.

Preparation of Mouse Rod Outer Segments—ROS were prepared from mouse retinas by using a discontinuous sucrose gradient centrifugation as described previously (2). Eight retinas from four mice were homogenized in 1.25 ml of ice-cold 47% sucrose solution containing 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl (pH 7.4) (buffer A). Retinal homogenates were transferred to 4.5-ml centrifuge tubes and sequentially overlaid with 1.5 ml of 37% and 1.0 ml of 32% sucrose dissolved in buffer A. The gradients were spun at 82,000 × g for 1 h at 4 °C. The 32:37% interfacial sucrose band containing ROS membranes was harvested and diluted with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA and centrifuged at 27,000 × g for 30 min. The ROS pellets were resuspended in 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA and stored at −20 °C. The non-ROS band designated as band II (37:47%) was also saved for comparison with ROS. Protein concentrations were determined by using the BCA reagent from Pierce, following the manufacturer’s instructions.

Caspase Activity Assay—Caspase-3 activity assay was performed with a caspase family colorimetric substrate kit II (BioVision, Mountain View, CA). Wild-type and IR knock-out mice were lysed, and the proteins were subjected to Western blot analysis with phospho-specific anti-IR/IGF-1R (Tyr(P)1158/Tyr(P)1162/Tyr(P)1163) antibody or used to directly measured the IR-associated PI3K activity.

RESULTS

Light Stress Activation of IR in Vivo—To determine whether light stress induced IR activation, we exposed rats to 5000 lux light for 3 h. After light exposure, rats were placed in the dark, and retinas were removed 0, 3, and 6 h later. Control experiments were done on overnight dark-adapted rats. The retinas were lysed, and the proteins were subjected to immunoprecipitation with anti-IRβ antibody followed by Western blot analysis with phospho-specific anti-IR/IGF-1R (Tyr(P)1158/Tyr(P)1162/Tyr(P)1163) antibody. Light stress resulted in the persistent activation of the IR at all time points analyzed (Fig. 1A), whereas the total IR content remained comparably constant (Fig. 1B), suggesting that light stress induced the activation of retinal IR in vivo.

Light Stress Induced the IR-associated PI3K Activity—We previously reported that tyrosine phosphorylation of the IR in the retina induced the activation of PI3K in the retina (3). To determine whether the light stress-induced activation of PI3K is regulated through the IR, we immunoprecipitated the IR from retina lysates prepared either from dark control or light-stressed (0 h) rats and measured PI3K activity. The PI3K activity associated with the IR in light-stressed retinas was greater than the PI3K activity in the IR from the dark-adapted controls (Fig. 1, C and D), suggesting that light stress induced the activation of PI3K, which is activated through its binding to the activated IR.

Light Stress Results in the Activation of Akt in Vivo—We (29) and others (1) have demonstrated that IR activation leads to the activation of Akt via PI3K in vitro. To determine whether light stress induces the activation of Akt, we subjected rats to light stress for 3 h at 5000 lux. After light exposure, rats were dark-adapted, and retinas were removed at 0, 3, and 6 h. Control experiments were done on overnight dark-adapted rats. Retinas were lysed, and the proteins were subjected to Western blot
analysis with anti-pAkt and Akt antibodies. Light stress resulted in the persistent activation of Akt in the retinas at all time points analyzed (Fig. 2). The activation of Akt was much higher immediately after light exposure (0 h) compared with 3 and 6 h (Fig. 2A). Total Akt content did not differ depending on time from exposure (Fig. 2B). The blot was reprobed with actin to ensure equal amounts of protein in each lane (Fig. 2C). These experiments clearly suggest that light stress induced the activation of Akt in vivo.

**Light Stress-induced, IR-associated PI3K Activity in Rod Photoreceptor Cells**—To determine whether the light stress-induced activation of PI3K is regulated through the IR in rod photoreceptor cells, we immunoprecipitated the IR from ROS that were prepared from retinas of both dark control and light-stressed (0 h) rats. We measured the PI3K activity and found significant PI3K activity associated with the IR in light-stressed ROS (Fig. 3, A and B). These results clearly suggest that light stress induced the activation of IR in ROS membranes.

**FIGURE 1.** Light stress activates the IR in vivo. Rats were dark-adapted overnight and then exposed to 5000lux for 3 h. At the end of the light exposure, rats were placed in the dark, and retinas were removed 0, 3, and 6 h later. Control experiments were done on overnight dark-adapted rats. The retinas were lysed, and equal amounts of proteins were subjected to immunoprecipitation with anti-IR antibody followed by Western blot analysis with the phospho-specific anti-IR/IGF-1R (Tyr(P)1158/Tyr(P)1162/Tyr(P)1163) antibody (A). The blot was stripped and reprobed with anti-IR antibody to ensure equal amounts of the IR were in each immunoprecipitate (B). Light stress-induced activation of the IR-associated PI3K activity. Retinal lysates from dark- and light-stressed (0 h) retinas were subjected to immunoprecipitation with anti-IR antibody followed by measuring the PI3K activity with PI-4,5-P2 and [γ-32P]ATP as substrates (C). The radioactive spots of PI-3,4,5-P3 were scraped from the TLC plate and counted (D). Data are mean ± S.D. for dark-adapted (dark) and light-stressed (LS) rats, n = 4; **, p < 0.001.

**FIGURE 2.** Light stress activates Akt in vivo. Rats were dark-adapted overnight and then exposed to 5000 lux for 3 h. After light exposure, rats were placed in the dark, and retinas were removed 0, 3, and 6 h later. Control experiments were done on overnight dark-adapted rats. The retinas were lysed, and equal amounts of protein were subjected to Western blot analysis with anti-pAkt (A) and Akt (B) antibodies. The blot was stripped and reprobed with anti-actin (C) antibody to ensure protein loading in each lane. Ratios of activated to total Akt (A/B) indicate increase in phosphorylation in light-stressed retinas. The activation level of control was set as 1.0 for comparison.

**FIGURE 3.** Light stress-induced activation of IR-associated PI3K activity in rod photoreceptor cells. Rod outer segments prepared from dark-adapted (dark) and light-stressed (LS) rats were solubilized and subjected to immunoprecipitation with anti-IR antibody followed by measuring the PI3K activity with PI-4,5-P2 and [γ-32P]ATP as substrates (A). The radioactive spots of PI-3,4,5-P3 were scraped from the TLC plate and counted (B). Data are means ± S.D. for dark-adapted (dark) and light-stressed (LS) rats, n = 4; *, p < 0.001.
Circulating Insulin Does Not Increase Activation of IRs When Light-stressed—From the results of this study, it appears that the IR pathway is activated in rod photoreceptor cells following light stress. It has been shown previously that there was a clear increase in IR phosphorylation and activation of a range of downstream signaling molecules in the retina following intravenous administration of insulin, and the response was because of insulin action in vascular endothelium (30). The possibility cannot be ruled out that some significant fraction of the signaling was because of insulin signaling in rod cells. In this study it is not clear whether the activated IR signaling in rods is because of light stress-induced increases in levels of circulating insulin. To determine whether light stress induces increases in the levels of circulating insulin, we measured serum insulin levels in both the dark-adapted and light-stressed rats. No difference was found in the levels of insulin between dark-adapted and light-stressed rats (supplemental Fig. S1). To further confirm these results, we measured blood glucose levels in both the dark-adapted and light-stressed rats and found no difference between dark-adapted and light-stressed rats (supplemental Fig. S1). Collectively, these data indicate that light stress does not increase the levels of circulating insulin.

Generation of the Conditional IR Knock-out Mice—We previously reported that the light-induced tyrosine phosphorylation of retinal IR leads to the activation of PI3K in rod photoreceptor cells (2). During light stress the IR in the rat retina was tyrosine-phosphorylated, which led to increased binding of PI3K (Fig. 1). Our data also indicate that the greater PI3K activity in rod photoreceptor cells is associated with IR activation (Fig. 3). To understand the significance of IR activation in rods under stressed conditions, we investigated the function of IR by using a genetic approach. Mice lacking IRS are born with normal features but develop early postnatal diabetes and die of ketoacidosis (15, 16). Therefore, a conditional IR disruption strategy was applied by mating rod-expressing Cre mice and IR floxed mices to generate conditional IR knock-out mice (Fig. 3). To determine whether light stress induces increases in the levels of circulating insulin, we measured serum insulin levels in both the dark-adapted and light-stressed rats. No difference was found in the levels of insulin between dark-adapted and light-stressed rats (supplemental Fig. S1). Collectively, these data indicate that light stress does not increase the levels of circulating insulin.

Assessment of the Effect of Opsin-Cre Transgene in the Deletion of IR in the Rod Photoreceptor Cells—To evaluate the efficiency of deletion of the IR in rod photoreceptors triggered by opsin-driven Cre expression, ROS membranes were prepared from homozygous conditional IR knock-out (cre+/−IRfloxed/floxed), IR hemizygous (cre+/−IR+/−floxed/floxed), and wild-type mice (IR+/+IR+/+floxed/floxed) and were subjected to Western blot analysis with anti-IRβ and anti-opsin antibodies. ROS membranes from IR knock-out mice generated with the 0.2-kg opsin-cre promoter had 50% less IR protein content than ROS membranes from control mice (Fig. 4, B–D). The results were similar with the 4.1-kg opsin-cre mouse line (data not shown). The residual IR protein might be attributable to a contamination from other retinal cells and/or incomplete deletion of the gene in some photoreceptor cells. To address the contamination, we used the exclusion marker, α1-Na+/−K+/−ATPase, which is mainly present in RPE cells, horizontal cells, ganglion cells, and Muller cells but not in photoreceptors (32). ROS and non-ROS (band II) membranes were prepared from mouse retinas by discontinuous sucrose gradient centrifugation and probed for the presence of α1-Na+/−K+/−ATPase. Fig. 4E shows that the α1 isoform was enriched non-ROS and some weak reactivity in ROS (Fig. 4E). We calculated the percentage of α1-Na+/−K+/−ATPase contamination was taken as 100%.

FIGURE 4. Generation of photoreceptor-specific IR knock-out mice. A, schematic diagram of loxP floxed IR loci. Rod photoreceptor-specific IR knock-out mice were generated by breeding mice with a floxed IR with mice that express Cre recombinase under the control of rod opsin promoter (0.2 or 4.1 kb). Primer pairs P1 and P2 were used to identify the wild-type and the floxed IR alleles. Rod outer segments were prepared from homozygous (cre+/−IRfloxed/floxed), hemizygous (cre+/−IR+/−floxed/floxed), and wild-type (IR+/+IR+/+floxed/floxed) mice. Equal amounts of protein were analyzed by Western blot analysis with anti-IRβ (B) or anti-opsin (C) antibodies. Opsin expression was used as an internal control. Quantitative analysis of bands of respective Western blot analysis was performed by using Kodak Image software (D). IRfloxed/floxed/Opsin ratio was set as 1.0 for comparison. Western blot analysis of α1-Na+/−K+/−ATPase expression in mouse ROS, and non-ROS fractions from wild-type (cre+/−IR+/+floxed/floxed), hemizygous (cre+/−IR+/−floxed/floxed), and homozygous (cre+/+IR+/+floxed/floxed) mice. Ten micrograms of ROS and non-ROS membranes were probed for α1-Na+/−K+/−ATPase (E) and opsin (F). Percentage of α1-Na+/−K+/−ATPase presence in ROS was calculated from the relative densities of α1-Na+/−K+/−ATPase from non-ROS membranes. The α1-Na+/−K+/−ATPase presence in non-ROS fraction was taken as 100%.
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In addition to opsin (Figs. 5F and 6F), we also examined the expression of another photoreceptor-specific protein, arrestin, the expression of which is also not altered in the ROS from either IR conditional knock-out mouse (Figs. 5, E and K, and 6, E and K), further suggesting the specificity of cell-specific deletion of the IR in the regulation of downstream effector molecules of the IR signaling pathway. Collectively, these results clearly suggest that decreased IR expression in ROS from both conditional IR knock-out mice leads to reduced recruitment of p85 and Akt survival factors to ROS membranes.

Specificity of Cell-specific Deletion of IR—To further assess the specificity of IR deletion or reduction in rod photoreceptor cells from the knock-out mice, we examined the expression of proteins involved in the IR signaling pathway in the non-ROS fraction (band II), which represents the retina minus the ROS membranes (2). The non-ROS fraction from the wild-type and conditional IR knock-out mice, which were generated with either the 0.2-kb (Fig. 5) or the 4.1-kb (Fig. 6) opsin-cre transgenic mice, were analyzed by using Western blot analysis with anti-IRβ, anti-p85, and anti-Akt antibodies. Densitometric analysis of immunoblots was performed in the linear range of detection, and absolute values were then normalized to photoreceptor-specific protein opsin (Figs. 5F and 6F). The levels of IR in ROS from both IR knock-out mouse lines were significantly less than the levels from wild-type controls (Figs. 5, A and G, and 6, A and G). The binding of the downstream effector molecules of the IR, the p85 subunit of PI3K (Figs. 5, B and H, and 6, B and H), and Akt (Figs. 5, C and I, and 6, C and I), was also significantly less in IR knock-out mouse ROS compared with the binding of these molecules from wild-type ROS, suggesting that IR regulates the PI3K and Akt activation in ROS. To determine the specificity of the reduced IR/PI3K/Akt survival factors in the ROS of IR conditional knock-out mice, we have examined the expression of an upstream regulator of IR, the protein-tyrosine phosphatase-1B (PTP-1B), which specifically dephosphorylates the IR. There was no significant difference in the expression of this protein in the ROS of the wild-type and either conditional IR knock-out ROS (Figs. 5, D and J, and 6, D and J).

Characterization of Photoreceptor-specific Conditional IR Knock-out Mice—We have reported previously that IR activation leads to the binding of p85 subunit of PI3K to ROS membranes (2). Because there is not much IR in the ROS from the knockdown ROS, we sought to determine whether there is any difference in the association of PI3K/Akt with ROS membranes after knocking down the IR in photoreceptors. Therefore, we examined the effect of the IR in the recruitment of PI3K and Akt survival factors to ROS membranes. ROS membranes from wild-type and conditional IR knock-out mice, which were generated with either the 0.2-kb (Fig. 5) or the 4.1-kb (Fig. 6) opsin-cre transgenic mice, were analyzed by using Western blot analysis with anti-IRβ, anti-p85, and anti-Akt antibodies. Densitometric analysis of immunoblots was performed in the linear range of detection, and absolute values were then normalized to photoreceptor-specific protein opsin (Figs. 5F and 6F). The levels of IR in ROS from both IR knock-out mouse lines were significantly less than the levels from wild-type controls (Figs. 5, A and G, and 6, A and G). The binding of the downstream effector molecules of the IR, the p85 subunit of PI3K (Figs. 5, B and H, and 6, B and H), and Akt (Figs. 5, C and I, and 6, C and I), was also significantly less in IR knock-out mouse ROS compared with the binding of these molecules from wild-type ROS, suggesting that IR regulates the PI3K and Akt activation in ROS. To determine the specificity of the reduced IR/PI3K/Akt survival factors in the ROS of IR conditional knock-out mice, we have examined the expression of an upstream regulator of IR, the protein-tyrosine phosphatase-1B (PTP-1B), which specifically dephosphorylates the IR. There was no significant difference in the expression of this protein in the ROS of the wild-type and either conditional IR knock-out ROS (Figs. 5, D and J, and 6, D and J).

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cre transgenic mice were examined for the expression of IR, p85, Akt, and PTP-1B. Densitometric analysis of immunoblots was performed in the linear range of detection, and absolute values were then normalized to actin. There was no significant difference among the expressions of the IR, p85, Akt, and PTP-1B in the non-ROS fraction from wild-type or either conditional knock-out mouse (supplemental Figs. S2 and S3). These experiments suggest that other cell types of the retina are not affected by the deletion, or reduction of the IR in photoreceptors does not affect the expression of IR and the recruitment of downstream effector molecules to ROS.

Effect of IR Reduction on Retinal Morphology and Function—Light microscopic examination of the retinas from left and right eyes of homozygous (cre−/+IR^flox/flox^), hemizygous (cre^+/−IR^flox/flox^), and wild-type (cre−/−IR^flox/flox^) mice at 6–8 weeks of age showed no difference in retinal structure among the three groups when each group was maintained in dim cyclic light (supplemental Fig. S4). The retinas appeared normal, and ROS appeared to be well organized (supplemental Fig. S4). There were 11 to 12 rows of photoreceptor nuclei in the ONL, the number usually observed for rodents without retinal degeneration (35). Quantitative analysis of the superior and inferior regions of the ONL layer showed no significant differences in the average ONL thickness measured at 0.25-mm intervals from the ONL to the inferior and superior ora serrata among the three groups (data not shown). There were no significant differences in the total retinal thickness in the superior and inferior regions of the retinas among the three groups (supplemental Fig. S4), indicating that rod photoreceptor viability was not different among these mice. We also tested three older animals (4–6 months of age) in each group and detected no greater loss of nuclei in the IR knock-out group (results not shown). Thus, mice lacking the IR in rod photoreceptors appeared to have no structural differences regardless of genotype. Homozygous (cre^−/−IR^flox/flox^), hemizygous (cre^+/−IR^flox/flox^), and wild-type (cre−/−IR^flox/flox^) mice did not exhibit any structural phenotype when maintained in dim cyclic light.

Electroretinography was used to evaluate photoreceptor function in the animal from each group. No differences among the animals were found in the amplitude of the a-wave of the scotopic electroretinography, which measures the response of rod photoreceptors to light stimuli, or the b-wave, which measures the response of the inner retinal cells. A_max and B_max, the maximal values of the a- and b-wave amplitudes at saturating light intensities, respectively, were calculated for each group, and no significant differences were found, indicating that the absence of the IR did not adversely affect the function of the retinas of mice born and raised in a relatively dim cyclic light environment (data not shown). These results clearly show that IR knock-out mice do not exhibit any functional phenotype when they are maintained in dim cyclic light.

Effect of IR on Retinal Structure after Light Stress—Figs. 1 and 3 show that light stress induced the activation of retinal IR; therefore, we tested the effect of loss of the IR in a light-damage model of retinal degeneration. For the light stress studies, we
used the conditional IR knock-out mice that were generated with the 0.2-kb mouse opsin promoter-controlled Cre mouse line (31). Homozygous (cre^{-/-}/IRflox/flox) mice were exposed to either 5000 lux for 5 days or 10,000 lux for 5 days. After a 7-day recovery period, we measured the extent of photoreceptor cell loss. Control mice were raised in dim cyclic light. Quantitative analysis of the superior and inferior regions of the ONL layer of unexposed control and homozygous IR knock-out mice showed no significant differences in the average ONL thickness measured at 0.25-mm intervals from the ONL to the inferior and superior ora serrata (Fig. 7B), indicating that there was no difference in rod photoreceptor viability between the two groups of mice (Fig. 7B). When they were exposed to 5000 lux (Fig. 7, C and E), the homozygous IR knock-out had significantly fewer rod photoreceptors in the superior and inferior regions than did wild-type mice. This was indicated by the loss of nuclei from the ONL. This loss was significantly enhanced in homozygous IR knock-out mice when they were exposed to 10,000 lux (Fig. 7, A, D, and E). Measurement of the ONL thickness along the vertical meridian confirmed the greater loss of rod photoreceptor nuclei in the IR knock-out mouse retinas compared with wild-type mouse retinas (Fig. 7, C and D). The superior retina of IR knock-out mice compared with their inferior retina was more susceptible to light damage (Fig. 7, C and D). The regional variation in the susceptibility to light damage is not known. These experiments clearly suggest that the IR is functionally important for photoreceptor survival.

Functional Evaluation of Photoreceptor Cells in IR Knock-out Mice after Light Damage—Seven days after light exposure, we evaluated the retinal function of the mice by using ERG. Fig. 8A shows typical wave forms from homozygous IR knock-out and wild-type mice before and after light stress. Mean amplitudes of a- and b-waves, respectively, from all mice tested per group are plotted as amplitude versus illumination (Fig. 8, B and C). No differences between the wild-type and IR knock-out group were

**FIGURE 7. Morphological analysis of IR knock-out mice after light stress.** Eyes from light-stressed wild-type (cre^{-/-}/IRflox/flox) and homozygous (cre^{+/+}/IRflox/flox) IR knock-out mice at 6–8 weeks of age were removed after light stress and were fixed, embedded in paraffin, and stained with hematoxylin and eosin. Representative sections from the superior and inferior retina in the light-stressed animals at 10,000 lux for 5 days are shown (A). INL, inner nuclear layer; ONL, outer nuclear layer; ROS, rod outer segment. Plots of ONL thickness at 0.25-mm intervals from the optic nerve head (ONH) along the vertical meridian in the superior and inferior regions of the retinas from mice that were exposed to either dim (B) or light stress at either 5000 lux (5k) (C) or 10,000 lux (10k) (D) for 5 days. E, quantification of morphologic changes. The average ONL thickness was calculated for the superior and inferior regions of the eye in wild-type and IR knock-out mice. Values are mean ± S.D., and the numbers of animals per group are indicated on top of each bar. *, p < 0.05; **, p < 0.01.
found in ERG amplitudes when the mice were maintained in dim cyclic light. There was a reduction in the amplitudes of both waves from both groups as a result of light stress; the greatest reduction occurred in the IR knock-out mice. Significant reductions in both α- and β-wave amplitudes were observed in IR knock-out mice that were exposed to 10,000 lux (Fig. 8, B and C). These data suggest that the absence of IR renders the photoreceptors of knock-out mice more sensitive to light damage than those of wild-type mice.

**DISCUSSION**

We showed previously that the important anti-apoptotic enzyme PI3K is regulated through light-activated IR in rod photoreceptor cells (2). In this study, we observed that light stress induced tyrosine phosphorylation of the IR in ROS membranes. We also observed increased IR-associated PI3K activity in light-stressed retinas. These observations led to the hypothesis that IR activation under stress conditions may be important for the survival and maintenance of photoreceptor structure and function. However, the functional roles of IR in rod photoreceptor cells and the mechanism for light-stress induced activation of the IR are not known. It was shown previously that IR phosphorylation and activation of a range of downstream signaling molecules in the retina increased following intravenous administration of insulin, and the response was because of insulin action in vascular endothelium (30). To rule out the possibility that light stress increased circulating insulin levels, we measured the insulin levels in dark- and light-stressed rats. Our data indicate that circulating insulin may not play a role in the light stress-induced IR activation. The IRS in ROS are localized to the plasma membrane (3), and insertion of the IR into the plasma membrane is necessary for hyperosmotic stress-induced receptor activation (38). It is possible that light stress-induced conformational changes in the plasma membrane result in the activation of the IR, but this remains to be determined.
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The IR/PI3K/Akt pathway provides survival signals in many tissues (39). We previously found that the IR is present in ROS membranes and can be activated in vivo in a light-dependent manner leading to binding of PI3K to the IR (2). Although the mechanism of activation of the IR is not known, regulation of its activity by light suggests that light-induced PI3K activity may mediate an innate neuroprotective mechanism. Because bright light stimulation causes the death of rod and cone photoreceptor cells (40–42), activation of the IR/PI3K/Akt pathway by relatively modest light levels may serve to prevent death of photoreceptors following bright light stress. We tested the hypothesis that the IR/PI3K/Akt pathway is neuroprotective in our conditional IR knockout mouse model. The IR knock-out mice have reduced IR/PI3K/Akt signaling compared with wild-type controls. The conditional IR knock-out mice were normal when maintained in dim cyclic light; however, exposure to bright light stress resulted in a retinal degeneration phenotype. The lack of phenotype in dim cyclic light could be due to residual expression of the IR, which may be protecting photoreceptor cells when exposed to dim cyclic light conditions. These results also suggest that a threshold of IR/PI3K/Akt signaling is required for normal photoreceptor survival. Under stress conditions, the IR/PI3K/Akt signal is compromised, which results in an increased sensitivity to light-induced photoreceptor degeneration. Our studies clearly suggest that the IR is functionally important for photoreceptor survival.

In this study we used two intensities of light stress (5000 and 10,000 lux) and found that the intensity of light stress is directly proportional to the reduction in retinal function and in photoreceptor number (ONL thickness). Under lower intensities we could not see a detectable structural or functional phenotype. Although the light stress condition used in this study was higher than that used for light-sensitive albino mice, our results are not surprising. LaVail et al. (43–45) demonstrated that different mouse strains exhibited a wide range of sensitivities to constant light exposure by using a light intensity of 1430 lux for up to 3 weeks. Albino C57BL/6J-c2j (B6al) mice showed considerable resistance to light damage in those studies (46). Furthermore, the Rpe65-Leu<sup>450</sup>Met variant increases retinal resistance against light-induced retinal degeneration by slowing down rhodopsin regeneration (46–48). Such possibility cannot be ruled out in this study. Higher light intensities were also required to obtain observable phenotypes in p53 knock-out mice and Bak/Bax double knock-out mice (49, 50). To ascertain photoreceptor integrity in this mouse line, retinal morphology and function were examined by hematoxylin and eosin-stained retinal sections and ERG in the 0.2-kb opsin-cre mice up to 8 months of age. Retinas were morphologically and functionally normal (31). Furthermore, we previously used the same opsin-driven Cre line for the generation of photoreceptor-specific Bcl-xl knock-out mice (8). These studies clearly suggest that Cre does not play a role in the structural and functional integrity of the retina in these knock-out mice. To further confirm that Cre has no deleterious effect on retinal structure and functional integrity, we generated photoreceptor-specific conditional PTP-1B knock-out mice for our experiments. Under the stress conditions, homozygous PTP-1B knock-out mice did not undergo stress-induced photoreceptor degeneration. These studies further provide assurance that Cre has no deleterious effects on photoreceptor structure and function.

IR activation reduced the activation of caspase-3 in R28 retinal neurons, and this effect was mediated through PI3K/Akt activation (1). Consistent with this study, we also observed the stress-induced activation of caspase-3 in IR knock-out mouse retinas. These studies clearly suggest that IR regulates the activation of caspase-3 through the PI3K/Akt pathway, and our studies clearly indicate the reduced recruitment of PI3K/Akt to ROS membrane of IR knock-out mice. Akt inhibits many pro-apoptotic targets by phosphorylating caspase-9, glycogen synthase kinase, bcl-2-associated death promoter, and members of the forkhead family transcriptional factors (51–58). Caspase-9

<sup>3</sup>R. V. S. Rajala, unpublished data.
is the master player of caspase protease cascade (52). The caspase-3 activation may be due to the impaired IR-activated Akt to phosphorylate caspase-9 (1). These results suggest that reduction of IR activation could lead to apoptosis mediated by caspase-3 activation in rod photoreceptors of IR knock-out mice.

In summary our data in this study demonstrate that a mouse with a specific deletion of the photoreceptor cell IR exhibits an increased sensitivity to light-induced photoreceptor degeneration. These findings provide direct evidence of a functional role for the IR in photoreceptor cell survival. Our results also suggest that the IR pathway is an important endogenous neuroprotective pathway in the rods, and IR activation may be an important element of photoreceptor neuroprotection. The photoreceptor-specific IR knock-out mouse line could further help to advance our understanding the role of IR in various photoreceptor functions.

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