Loss of BCL-X<br>in Rod Photoreceptors: Increased Susceptibility to Bright Light Stress

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PURPOSE. BCL-XL, an anti-apoptotic member of the BCL-2 family proteins and a cell death/survival checkpoint regulator, was shown to be upregulated in bright light-stressed mouse photoreceptors during an investigation of bright light-induced protein expression. To investigate the significance of BCL-XL upregulation in the bright light damage model, the Bcl-x gene was disrupted specifically in mouse rod photoreceptors, and the effect of Bclx disruption was characterized on retinal apoptosis, function, and morphology.

METHODS. Rod-specific Bcl-x knockout mice, generated by mating mouse opsin promoter–controlled Cre mice with floxed Bcl-x mice, were subjected to bright light stress. Retinal apoptosis in the bright light–stressed conditional Bcl-x knockout mice was characterized with TUNEL, DNA fragmentation, and nuclear staining assays. Photoreceptor structural and functional integrity in the bright light–stressed conditional Bcl-x knockout mice was determined by measuring photoreceptor outer nuclear layer (ONL) thickness and electroretinography amplitudes.

RESULTS. Disruption of Bclx in rod photoreceptors caused increased photoreceptor apoptosis, decreased retinal function, and decreased ONL thickness in bright light–stressed mice.

CONCLUSIONS. The loss of BCL-XL increased rod photoreceptor susceptibility to bright light stress. Although the biochemical mechanism(s) of BCL-XL in photoreceptor death or survival has not been investigated extensively, results of the present study suggest that BCL-XL, a cell survival/death checkpoint regulator, is involved in photoreceptor survival under bright light stress. (Invest Ophthalmol Vis Sci. 2006;47:5583–5589) DOI:10.1167/iovs.06-0163

One of the fundamental questions in the search for pathogenic mechanisms of neurodegenerative diseases is how mammalian neurons survive under normal or stressed conditions. It has been shown that the survival of postmitotic neurons can be achieved through balanced levels of death/survival checkpoint regulators, such as the BCL-2 family, which includes a group of anti-apoptotic and pro-apoptotic proteins.1–4 Emerging evidence suggests that phosphoinositide 3-kinase (PI3K) and its product, phosphatidylinositol-3,4,5-triphosphate (PIP3), is, at least in part, responsible for the survival of neurons through growth factor receptor-mediated activation of AKT, a serine-threonine kinase.5–7 As a result of AKT activation, BCL-XL exerts its function through a number of alternative pathways, depending on cell type, and acts as a checkpoint regulator for survival.8–10 Overexpression of BCL-XL in photoreceptors was shown to protect against lead-induced apoptosis in mice through a blockage of pro-apoptotic protein (BAX)–mediated mitochondrial contact.1 However, overexpression of BCL-XL did not appear to rescue either the inherited retinal degeneration caused by an Rd/Rd mutation or a dominant rhodopsin gene mutation.11 To understand the functional significance of BCL-XL upregulation in bright light–stressed photoreceptors and to circumvent the embryonic lethality of a germline Bclx disruption, we disrupted the Bcl-x gene specifically in mouse rod photoreceptors using a Cre/loxP-based conditional knockout strategy (for review of the technology, see Le and Sauer12). In this strategy, rod-expressing Cre mice and floxed Bcl-x (loxP-flanked) mice are required to make the rod-specific Bcl-x knockout mice. Rod-expressing Cre mice were generated with mouse opsin promoter–directed cre transgenics.13 The rod-expressing Cre mice were mated with the existing loxP-flanked Bcl-x (floxed) mice to generate the rod-specific Bcl-x knockout (KO) mice.14 We report herein the phenotypic characterization of the rod-specific Bcl-x KO mice.

MATERIALS AND METHODS

Generation of Rod Photoreceptor–Specific Bcl-x Knockout Mice

All experiments with animals conformed to the guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center, the Dean A. McGee Eye Institute, and the Oklahoma Medical Research Foundation.

Conditional Bcl-x KO mice were generated by mating the floxed Bcl-x mice with the short mouse opsin promoter–controlled Cre (SMOPC1) mice to obtain albino conditional Bcl-x KO mice.13,14 To minimize the influence of genetic background in our study, littermates were used as hemizygotes (HEMI) and wild-type (WT) controls throughout this study. Genotyping of WT, HEMI, and KO Bcl-x mice was performed according to the conditions described previously14 using primer pairs a (5′-GCC ACC TCA TCA GTC GGG GTG AG-3′) and b (5′-TCA GAA GCC GCA ATA TTC CCT AT-3′) to detect a 160-bp product for the WT allele and a 195-bp product for the floxed Bcl-x allele. PCR diagnosis for cre was performed according to the procedures described by Le and Sauer12 using primer pairs c (5′-AGG TGT AGA GAA GGC ACT TAG C-3′) and d (5′-CTA ATC GCC ATC TTC CAG CAG G-3′) to detect a 411-bp product.

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Mouse Model of Bright Light Stress

Eight-week-old albino mice were exposed to white light at an illumination of 7000 lux for 48 hours. The temperature in the light box was maintained at 25°C. Mice were maintained in 100-lux cyclic light after exposure to bright light. Gene expression, apoptosis, and DNA fragmentation assays were carried out 24 hours after the light exposure. Morphologic and functional analyses were carried out 7 days after the bright light exposure.

Retinal Morphology

After euthanatization, the eyes were dissected and fixed at room temperature overnight in 4% paraformaldehyde, 20% isopropanol, 2% trichloroacetic acid, 2% zinc chloride. Fixed eyes were then paraffin embedded and sectioned (5-μm thickness). Sections were stained with hematoxylin and eosin (H&E), and the thickness of the outer nuclear layer (ONL) was measured at a distance of 240 μm from the optic nerve to the inferior and superior ora serrata. Results were plotted as mean ± SD. Differences were assessed by ANOVA or t test, depending on the experimental design. *P < 0.05 was considered significant.

Immunohistochemistry and Western Blot Analysis

Immunostaining of BCL-XL was performed according to established procedures15 using a polyclonal anti–BCL-XL antibody (H62: 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit IgG secondary antibody (Alexa Fluor 488 conjugated; Molecular Probes, Eugene, OR). For nuclear staining, 4,6-diamidino-2-phenylindole-2-HCI (DAPI) and propidium iodide (PI) mounting media were used according to manufacturer’s instruction (Vector, Burlingame, CA). For immunoblotting of BCL-XL, the polyclonal anti-BCL-XL antibody (H62: 1:200 dilution; Santa Cruz Biotechnology) and the peroxidase-linked anti-rabbit IgG secondary antibody (1:5000 dilution; Amersham Biosciences, Buckinghamshire, England) were used. For immunoblotting of β-actin as a loading control, the anti-actin monoclonal antibody (MA1–744: 1:100 dilution; Affinity Bioreagents, Golden, CO) and the peroxidase-linked anti-mouse IgG secondary antibody (1:5000; Amersham Biosciences) were used. Images were detected by chemiluminescence with a substrate (Super Signal West Dura Extended Duration Substrate; Pierce, Rockford, IL) and an imaging system (Molecular Image Station 4000R; Eastman Kodak, Rochester, NY) and analyzed with imaging software (Molecular Imaging Software version 4.0.0; Eastman Kodak).

Electroretinography

Photoreceptor function was assessed through electroretinography (ERG). Scotopic and photopic electroretinograms were measured (UTAS-E 5000 ERG System, LKC Technologies, Inc., Gaithersburg, MD), according to the conditions described previously.15–17

TUNEL Staining

TUNEL assay was performed (In Situ DNA Fragmentation Assay Kit; BioVision, Mountain View, CA) to detect cleaved DNA in the paraffin-embedded sections, according to the manufacturer’s instruction.

DNA Fragmentation

Mice were humanely killed 24 hours after the bright light stress. Retinas were removed and homogenized in 100 mM Tris-HCl (pH 8.0) containing 20 mM EDTA, 0.8% SDS, and proteinase K (20 μg/ml), and the mixture was incubated overnight at 55°C. After phenol-chloroform extraction of the mixture, the DNA was precipitated with ethanol, washed with 75% ethanol, dissolved in TE buffer, and fractionated on a 2% agarose gel.

RESULTS

Generation of the Conditional Bcl-x Knockout Mice

BCL-XL is considered a predominant BCL-2 family protein in the retina and is expressed in the adult mouse retina.18,19 During the analysis of light stress–regulated protein expression in the mouse retina, BCL-XL was shown to be upregulated in the retina and in photoreceptor cells after bright light stress (see Figs. 2, 3). To understand the significance of BCL-XL upregulation in the rods under stressed conditions, we investigated the function of BCL-XL through a genetic approach. Given that germline Bcl-x disruption caused embryonic lethality in mice,20 a conditional Bcl-x disruption strategy was applied by mating the existing rod-expressing Cre mice and the floxed Bcl-x mice to generate the conditional Bcl-x KO mice. The floxed Bcl-x mouse line was previously generated for studies on the role of primordial germ cells in embryogenesis.14 In this mouse line, exons 1 and 2 are flanked by two loxP sites (Fig. 1). For this study, we generated short (0.2 kb) and long (4.1-kb) mouse opsin promoter-controlled Cre mouse lines named SMOPC1 and LMOPC1, respectively. Both SMOPC1 and LMOPC1 expressed functional Cre recombinase in the rod photoreceptors across the retina, and the structure and function of the photoreceptor cells were normal until mice were at least 8 months of age.15 In our preliminary characterization of the rod photoreceptor-specific Bcl-x KO mice generated with either SMOPC1 or LMOPC1 mice, we observed similar phenotypic changes in the retina of the KO mice (data not shown). Because the SMOPC1 mice produced relatively less Cre and thus the effect
sections of the conditional Bcl-x KO mice. Our initial examination of conditional Bcl-x KO and control mice born and raised in a 100-lux cyclic light environment did not detect TUNEL-positive cells in the retinas (Fig. 4). However, exposure to bright light (7000 lux) for 48 hours induced extensive photoreceptor cell apoptosis, as indicated by TUNEL-positive staining in the ONL (Fig. 4). In comparison with control retinas, a significant increase was observed in the number of TUNEL-positive cells in the ONL of the bright light–stressed conditional Bcl-x KO mice (Fig. 4), suggesting that the loss of Bcl-x contributed to the increased apoptosis in rod photoreceptors, presumably because of the loss of the anti-apoptotic activity of BCL-XL. DNA fragmentation assay showed that bright light stress promoted more DNA fragmentation, a typical characteristic of apoptosis, in all types of mice (Fig. 5). However, more DNA fragmentation was observed in the conditional Bcl-x KO mice than in the HEMI and WT mice after exposure to bright light (Fig. 5), suggesting more apoptosis in the conditional Bcl-x KO mice. Finally, significantly more condensed nuclei, an indication of condensed chromatin and characteristic of apoptosis,21 were present in the conditional Bcl-x KO mice (Fig. 6).

**Effect of BCL-XL on Retinal Morphology**

Examination of retinal morphology of at least 20 eyes from 2-month-old Bcl-x KO mice demonstrated no detectable change in the retinal morphology of the Bcl-x KO mice under normal conditions (Fig. 7), indicating that the loss of BCL-XL had no apparent effect on retinal structure in 2-month-old Bcl-x KO mice under normal conditions. Morphologic analysis also showed that there was no detectable change in the retinas of 6-month-old conditional Bcl-x KO mice (data not shown). Figure 7 summarizes a comparison of ONL thickness among 48 eyes of the 2-month-old conditional Bcl-x KO (cre+/−/Bcl-x−/−) mice, 28 eyes of HEMI (cre+/−/Bcl-x+/−) mice, and 26 eyes of WT (cre+/−/Bcl-x+/+) mice after the light stress. Statistical analysis indicated that the differences in ONL thickness between the conditional Bcl-x KO mice and the two types of littermate controls were significant. As expected, no significant differences in ONL thickness were observed between HEMI and WT mice. Again, our results suggested that the loss of BCL-XL contributed to the increased susceptibility of light-induced rod photoreceptor degeneration.

**Effect of BCL-XL on Photoreceptor Function**

To determine whether the disruption of Bcl-x in the rod photoreceptor affected photoreceptor function, ERG was performed on 2-month-old conditional Bcl-x KO mice. No significant differences in scotopic or photopic electroretinograms were detected between the Bcl-x KO mice and WT littermate controls on analysis of ERG recordings from at least 20 eyes of the Bcl-x KO mice (data not shown), suggesting that BCL-XL is not required for the maintenance of photoreceptor function under normal conditions. ERG analysis also suggested that there was no detectable loss of photoreceptor function in the 6-month-old conditional Bcl-x KO mice (data not shown). However, significant differences in a-wave and b-wave amplitudes of the scotopic electroretinograms were detected after 2-month-old conditional Bcl-x KO mice and WT littermates were subjected to bright light stress. Figure 8 summarizes a comparison of ERG recordings among 48 eyes of 2-month-old homozygous conditional Bcl-x KO (cre+/−/Bcl-x−/−) mice, 28 eyes of HEMI (cre+/−/Bcl-x+/−) mice, and 26 eyes of WT (cre+/−/Bcl-x+/+) mice after light stress. Statistical analysis of the scotopic ERG recordings indicated that the differences in a-wave and b-wave amplitudes between the Bcl-x KO mice and the two types of littermate controls were significant.
significant differences in the scotopic electroretinograms were observed between the HEMI and WT mice. As expected, no differences were observed in the photopic electroretinograms of conditional Bcl-x KO mice and WT littermate controls (Fig. 8). In summary, our results suggest that the loss of BCL-X L contributed to the increased susceptibility of bright light–induced damage in rod photoreceptors, which, in turn, caused the loss of rod photoreceptor function.

**DISCUSSION**

In this study, we initially used a light intensity of 5000 lux for 5 hours that caused the apoptosis of most photoreceptors in light-sensitive Balb/c mice. However, this light intensity did not cause significant changes in retinal morphology and function in our albino Bcl-x KO mice or their WT littermates, suggesting that the genetic background of our albino mice was more resistant to this light stress condition. The actual light stress condition (7000 lux for 48 hours) used in this study was a result of a titration that allowed the detection of light-induced photoreceptor damage in all types of mice and of the observation of a phenotypic difference between WT and conditional Bcl-x KO mice. Although the light-stress condition used in this study was higher than that used for light-sensitive albino Balb/c mice, our results are not surprising. LaVail et al. 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 C57B6 mice showed...
considerable resistance to light damage in those studies. Higher light intensities were also required to obtain observable phenotypes in the studies of p53 knockout mice and Bak/Bax double knockout mice. Given that part of the genetic background in our mice was derived from FVB/n, which has not been well investigated for its susceptibility to bright light, it is possible that the FVB/n background may also play a role in light resistance. For these reasons, we used WT, HEMI, and KO littermates.

Although the fate of light-induced and hereditary retinal degeneration is photoreceptor death, fundamental differences exist between the upstream events and the downstream pathways. Using rhodopsin and transducin KO mice to shut off the phototransduction signal, Hao et al. demonstrated the presence of at least two independent apoptotic pathways for light-induced photoreceptor degeneration. Low light intensity induced phototransduction-related apoptosis and high light intensity triggered transcription factor AP-1–induced apoptosis.

**FIGURE 5.** Analysis of apoptosis with DNA fragmentation assay. M, 100-bp DNA molecular weight markers. Lanes 1 to 6: Retinal DNA from bright light–stressed or normal light–exposed conditional Bcl-xL KO, HEMI, and WT mice 24 hours after exposure to bright light. Bright light caused DNA fragmentation in all groups of mice; however, greater DNA fragmentation was detected in the retinas of the bright light–stressed conditional Bcl-xL KO mice.

**FIGURE 6.** Analysis of apoptosis with propidium iodide (PI) nuclear staining assay. (A–C) Retinal sections of bright light–stressed conditional Bcl-xL KO, HEMI, and WT mice. (D) Retinal section of normal light–exposed conditional Bcl-xL KO mice. Retinal sections were prepared 24 hours after bright light exposure. (E–G) Enlarged areas of A1, B1, and C1. Arrows indicate nuclei with condensed chromatin, a characteristic of apoptotic nuclei. Scale bars: 20 μm. (H) Average numbers of condensed nuclei in the ONL of the superior region. Numbers were derived from fluorescence microscope images of the PI-stained sections from at least three mice. Error bars represent SD. Significantly more apoptotic photoreceptor nuclei (arrows) were found in the bright light–stressed conditional Bcl-xL KO mice.

BCL-XL Loss and Light Stress to Rod Photoreceptors... Although the molecular mechanism of BCL-XL in photoreceptor survival has not been well investigated, BCL-XL is considered a downstream target of the PI3K-AKT pathway and a cell death/survival checkpoint regulator of mitochondrial dysfunction. In our study, BCL-XL expression in the photoreceptors was upregulated after WT mice were subjected to a 48-hour period of bright light stress. To determine the functional significance of bright light–induced BCL-XL upregulation, a genetic approach was used to disrupt the Bcl-xL gene in photoreceptors. Our results demonstrate that the loss of BCL-XL, a potential photoreceptor death/survival checkpoint regulator, contributed to increased photoreceptor susceptibility to bright light stress. However, the protective effect of BCL-XL may only be detected in mice with a genetic background resistant to lower light intensity–induced apoptosis. This may account for the discrepancy between our study and the study of Joseph and Li, in which lower light intensity caused a severe loss of the photoreceptor ONL in the mice overexpressing BCL-XL, suggesting that the photoreceptor degeneration in these mice was likely a consequence of lower light intensity–induced apoptosis that could circumvent the detection of the protective effect of BCL-XL under more stringent light intensities. Alternatively, BCL-XL may only exert its protective effect under high light intensities. A similar light intensity did not cause morphologic or functional changes in our mice, indicating that the mechanisms of photoreceptor apoptosis might be different in these two studies. A recent study may provide some mechanistic insights. Donovan et al. demonstrated that pro-apoptotic BCL-2 family proteins were downregulated during postnatal development and were not upregulated under a low light intensity that caused photoreceptor degeneration in the light-sensitive Balb/c mice. Their results suggest that the BCL-XL may not be needed as a gatekeeper for mitochondria under this lower light intensity–induced stress. The difference in genetic backgrounds and mechanisms of light-induced apoptosis may dictate the role of BCL-XL in photoreceptor survival and our ability to observe its protective effect under light stress.

Because our study was related to retinal degeneration, we used Smopc1 mice, which expressed a low level of Cre recombinase in rods to generate the conditional Bcl-xL KO mice. To ascertain photoreceptor integrity in this mouse line, retinal morphology and function were examined in H&E-stained retin...
nal sections and were measured by ERG in the SMOPC1 mice up to 8 months of age. Retinas were morphologically and functionally normal. Additional assurance of retinal integrity was provided by including littermate hemizygous conditional Bcl-x KO (cre+/– Bcl-xf/–) mice as controls. Therefore, any potential problems associated with cre expression could be detected. As expected, these cre+/– mice demonstrated normal phenotypes similar to those of the cre–/– mice, suggesting that Cre did not play a role in structural and functional changes in the conditional Bcl-x KO mice.

**FIGURE 7.** Retinal morphology in the conditional Bcl-x knockout mice. (A–F) Representative results of H&E-stained retinal sections from bright light-stressed or normal light-exposed conditional Bcl-x KO, HEMI, and WT mice. Retinal sections were prepared 1 week after light exposure. Scale bar: 20 μm. (G) ONL thickness of the conditional Bcl-x KO and control mice. (H) Average ONL thickness of the bright light-stressed conditional KO and control mice. Error bars represent SD. No apparent difference in ONL thickness was observed among the normal light-exposed mice (D–F). Loss of ONL thickness was observed in all bright light-stressed mice (A–C); however, significantly more loss in ONL thickness was observed in the bright light–stressed Bcl-x KO mice (A).

**FIGURE 8.** Retinal function in the bright light–stressed conditional Bcl-x knockout mice. (A) Representative scotopic ERGs from the conditional Bcl-x KO mice and WT littermate controls. Amplitudes are indicated as the difference between the baseline and the peak. (B, C) Average scotopic and photopic ERG amplitudes from at least 20 eyes of the conditional Bcl-x KO, HEMI, and WT mice. ERG was performed 1 week after the bright light exposure. Error bars represent SD. Significant loss of rod function was observed in the bright light–stressed conditional Bcl-x KO mice. No detectable loss of cone function was observed in the conditional Bcl-x KO mice.
A subset of rod bipolar cells also expressed Cre in the SMOPC1 mice. Disruption of Bcl-x in the rod bipolar cells was unlikely to have caused the observed phenotypic changes in our conditional Bcl-x KO mice because no detectable BCL-XL expression was observed in rod bipolar cells under normal (non-light-stressed) and light-stressed conditions, bipolar cells are not known for light-induced damage, and no detectable loss of retinal function was observed in the conditional Bcl-x KO mice under normal conditions, as judged by ERG. To further confirm that the disruption of Bcl-x in the rod bipolar cells did not cause the observed phenotypes in our conditional Bcl-x KO mice, we used a line of Cre mice (LMOPC1) that did not express Cre in rod bipolar cells to generate the rod-specific Bcl-x KO mice. In these mice, morphologic and functional changes were similar to what was observed in the conditional Bcl-x KO mice generated with the SMOPC1 mice (data not shown). This result confirms that the disruption of Bcl-x in the rod photoreceptors caused the functional and morphologic changes in the retina of the conditional Bcl-x KO mice.

In summary, our results demonstrate that BCL-XL plays a critical role in photoreceptor susceptibility to bright light-induced stress through an anti-apoptotic mechanism. Our results are consistent with the finding that deficiency of the pro-apoptotic proteins BAX and BAK protected photoreceptors from light damage in mice. This study is part of our long-term effort to identify the roles of lipid second messengers and their downstream targets in protecting photoreceptors from stress-induced degeneration. We are investigating the role of insulin receptor activation of the PI3K and AKT pathways in rod photoreceptors. The combined results of these studies will provide a better understanding of receptor-activated photoreceptor survival and of the feasibility of using survival pathway intermediates in clinical interventions for retinal degeneration.

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References