

Müller Cell Response to Blue Light Injury of the Rat Retina

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PURPOSE. In addition to photoreceptor degeneration, excessive light causes degenerative alterations in the inner retina and ganglion cell death. A disturbance in osmohomeostasis may be one causative factor for the alterations in the inner retina. Because Müller cells mediate inner retinal osmohomeostasis (mainly through channel-mediated transport of potassium ions and water), the authors investigated whether these cells alter their properties in response to excessive blue light.

METHODS. Retinas of adult rats were exposed to blue light for 30 minutes. At various time periods after treatment, retinal slices were immunostained against glial fibrillary acidic protein and potassium and water channel proteins (Kir4.1, aquaporin-1, aquaporin-4). Patch-clamp recordings of potassium currents were made in isolated Müller cells, and the swelling of Müller cell bodies was recorded in retinal slices.

RESULTS. After blue light treatment, Müller cells displayed hypertrophy and increased glial fibrillary acidic protein. The immunostaining of the glial water channel aquaporin-4 was increased in the outer retina, whereas the immunostaining of the photoreceptor water channel aquaporin-1 disappeared. Blue light treatment resulted in a decrease and a dislocation of the Kir4.1 protein in the whole retinal tissue and a decrease in the potassium conductance of Müller cells. Hypo-osmotic stress evoked a swelling of Müller cell bodies in light-treated retinas that was not observed in control tissues.

CONCLUSIONS. The decrease in functional Kir channels may result in a disturbance of retinal potassium and water homeostasis, contributing to the degenerative alterations of the inner retina after exposure to blue light. (*Invest Ophthalmol Vis Sci.* 2008;49:3559–3567) DOI:10.1167/iovs.08-1723

Photoreceptor cell death and damage to the pigment epithelium are characteristic events after exposure to excessive light. It has been shown that, in particular, short-wave-

length blue light heavily damages the retinal tissue.^{1–3} Blue light as a component of the light of ophthalmologic instruments contributes to the development of macular edema after surgery; blue light is responsible for solar retinitis and plays a role in the pathogenesis of age-related macular degeneration.⁴ In animal models of light-evoked retinal degeneration, the death of photoreceptor cells is caused predominantly by apoptosis.^{3,5} Photoreceptor cell death is often accompanied by the development of local edema in the outer retina because of the breakdown of the outer blood-retinal barrier and normotonic shrinkage of the cells that undergo apoptosis.^{1,6} The volume decrease of apoptotic cells occurs through channel- and transporter-mediated efflux of osmolytes, especially of potassium and chloride ions; the ion efflux creates an osmotic gradient that draws water out of the cells.^{7–9} In addition to the damage of the outer retina, excessive light may cause degenerative alterations in the inner retina.¹⁰ In a rat model of excessive light exposure, retinal ganglion cells underwent apoptosis.¹¹ The mechanisms of light-evoked degenerative alterations in the inner retina are unclear.

Retinal light injury is accompanied by local edema in the subretinal space and outer retina. Normally, the osmohomeostasis of the outer retina is regulated by photoreceptor cells and the pigment epithelium, at least in part by water flux through aquaporin-1 water channels.^{12,13} Ion and water homeostasis of the inner retina and outer plexiform layer is regulated by Müller glial cells.¹⁴ Water flux across Müller cell membranes is facilitated by aquaporin-4 water channels,¹⁵ and spatial buffering of potassium currents flowing through Müller cells is mediated by inwardly rectifying potassium (Kir) channels, especially of the Kir4.1 subtype.¹⁶ Colocalization of Kir4.1 and aquaporin-4 proteins in distinct membrane domains of Müller cells has led to the suggestion that water transport is coupled to potassium currents through Müller cells.¹⁵

It has been shown in animal models that retinal ischemia-reperfusion and inflammation are accompanied by a decrease in the potassium conductance of Müller cells and a disturbance of the rapid water transport across Müller cell membranes.^{17–19} The disturbance in transmembrane water transport is indicated by an induction of cellular swelling under hypo-osmotic conditions not observed in control tissues. Impairment in potassium and water transport through Müller cells, which may result in neuronal hyperexcitation and tissue edema, contributes to degenerative alterations in the inner retina.²⁰ Retinal light injury is mediated, at least in part, by pathogenic factors similar to those in retinal ischemic and inflammatory injuries (e.g., oxidative stress induced by a prolonged increase in oxygen tension and photooxidation,^{21,22} inflammatory responses triggered by the release of proinflammatory cytokines and polyunsaturated fatty acids such as arachidonic acid^{3,23,24}). Therefore, we investigated in the present study whether Müller cells respond to blue light injury of the retina with alterations in the localization of potassium and water channels. We found that blue light treatment of rat retinas results in an upregulation of aquaporin-4 in the outer retina, a dislocation of Kir4.1 protein, and a decrease in the potassium conductance of Müller cells.

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ler cells; the inactivation of Kir channels is accompanied by an alteration in the osmotic swelling characteristics of the cells. The results suggest a disturbance of Müller cell-mediated potassium and water homeostasis in the retina after exposure to blue light, which may contribute to the degenerative alterations in the inner retina.

MATERIALS AND METHODS

Materials

Chloromethyltetramethylrosamine (Mitotracker Orange) was purchased from Molecular Probes (Eugene, OR). Triamcinolone acetate (9 α -fluoro-16 α -hydroxyprednisolone), prostaglandin E₂ (PGE₂), 4-bromophenacyl bromide, indomethacin, dithiothreitol, and all other substances used were obtained from Sigma-Aldrich (Taufkirchen, Germany). The following antibodies were used: mouse anti-glutamine synthetase (1:250; Chemicon), mouse anti-glia fibrillary acidic protein (GFAP; 1:200; G-A-5 clone; Sigma), rabbit anti-Kir4.1 (1:200; Alomone Laboratories, Jerusalem, Israel), rabbit anti-rat aquaporin-4 (1:200; Sigma), rabbit anti-aquaporin-1 (1:200; Chemicon), Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova, Hamburg, Germany), and Cy2-coupled goat anti-mouse IgG (1:200; Dianova).

Light Treatment

All procedures concerning animals were conducted in accordance with applicable German laws and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were reared in 12-hour light/12-hour dark (6:00 AM-6:00 PM) cycles with 60 to 100 lux within the cages. One eye each of adult pigmented Long-Evans rats (weight range, 250–300 g) was exposed to blue light (405 \pm 10 nm; 30 minutes; 8 mW/cm² at the level of the cornea) with the use of a stereomicroscope (Lumar V12; Zeiss, Oberkochen, Germany). After dark adaptation for 30 minutes, anesthesia was induced by intramuscular ketamine (100 mg/kg body weight) and xylazine (5 mg/kg), and the pupils were dilated using tropicamide (5 mg/mL; Midrum; Ankerpharm, Rudolstadt, Germany). Animals were killed by carbon dioxide 1, 2, and 3 days after light treatment, and the eyes were removed. Light treatment resulted in damage of a circular retinal area with a diameter of approximately 3 mm.

Electrophysiological Recordings

For electrophysiological and cell-swelling experiments, cells and slices from injured retinal areas and from noninjured areas of the treated eyes (approximately 4 mm distant from the edges of the injured areas) were investigated, as were cells from untreated control eyes. The experiments were conducted at room temperature (22°C–25°C) or, when indicated, at 35°C to 37°C. Whole-cell patch-clamp recordings were carried out using Müller glial cells acutely isolated in papain and DNase I-containing solutions, as described previously.^{18,19} Cell suspensions were stored in serum-free minimum essential medium (Sigma-Aldrich) at 4°C (up to 6 hours) before use. Voltage-clamp recordings were performed with the use of an amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) and a computer program (ISO-2; MFK, Niedernhausen, Germany). Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) and had resistances between 4 and 6 M Ω when filled with an intracellular solution containing 10 mM NaCl, 130 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES-Tris (pH 7.1). Signals were low-pass filtered at 1, 2, or 6 kHz (8-pole Bessel filter) and digitized at 5, 10, or 30 kHz, respectively, with the use of a 12-bit A/D converter. The recording chamber was continuously perfused with extracellular solution consisting of 135 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 10 mM HEPES, and 11 mM glucose (pH 7.4). To evoke potassium currents, depolarizing and hyperpolarizing voltage steps of 250-ms duration, with increments of 10 mV, were applied from a holding potential of -80 mV. The membrane capacitance of the cells

was measured by the integral of the uncompensated capacitive artifact (filtered at 6 kHz) evoked by a hyperpolarizing voltage step from -80 to -90 mV when barium chloride (1 mM) was present in the bath solution to block the potassium currents through Kir channels. To isolate fast transient (A-type) potassium currents, two voltage step protocols were used in the presence of barium chloride (100 μ M): depolarizing steps (1) after maximal activation of these currents by a 500-ms prepulse to -120 mV and (2) after steady state inactivation of the currents by a 500-ms prepulse to -40 mV. Currents obtained with both protocols were subtracted (1 $-$ 2); if present, A-type currents became visible, whereas delayed rectifier currents were eliminated. The resting membrane potential was measured in the current-clamp mode.

Müller Cell Swelling

To determine the volume changes of Müller glial cells evoked by hypo-osmotic stress, the cross-sectional area of Müller cell somata in the inner nuclear layer of each retinal slice was measured.¹⁷ Retinal slices (1-mm thick) were placed in a perfusion chamber and were loaded with the vital dye chloromethyltetramethylrosamine (10 μ M; Mitotracker Orange; Molecular Probes). It has been shown that this dye is taken up selectively by Müller cells in the retina, whereas neurons, astrocytes, and microglial cells remain unstained.²⁵ The stock solution of the dye was prepared in dimethyl sulfoxide and resolved in extracellular solution that contained 136 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-Tris, and 11 mM glucose (pH 7.4). A gravity-fed system with multiple reservoirs was used to perfuse the recording chamber continuously with extracellular solutions. The hypo-osmotic solution was added by rapidly changing the perfusate. The bathing solution in the recording chamber was totally changed within 1 minute of perfusion. The hypo-osmolar solution contained 60% of control osmolarity and was composed by the addition of distilled water to the extracellular solution. Barium chloride (1 mM) was preincubated for 10 minutes in extracellular solution before it was applied within the hypo-osmotic solution. Blocking substances were preincubated for 15 minutes before hypo-osmotic challenge. Slices were examined using an upright confocal laser scanning microscope (LSM 510 Meta; Zeiss) and a 63 \times /0.9 water-immersion objective (Achromplan; Zeiss). The pinhole was set at 172 μ m; the thickness of the optical section was adjusted to 1 μ m. Chloromethyltetramethylrosamine (Mitotracker Orange; Molecular Probes) was excited at 543 nm, and emission was recorded with a 560-nm long-pass filter. The vital dye-stained cell somata in the inner nuclear layer were recorded at the plane of their maximal extension. To ensure that the maximum soma areas were precisely recorded, the focal plane was continuously adjusted in the course of the experiments. Stained cell bodies from the surfaces of the slices to a depth of approximately 20 μ m were recorded.

Immunostaining

Isolated retinas were fixed in 4% paraformaldehyde for 2 hours. After several washing steps in buffered saline, the tissues were embedded in saline containing 3% agarose (wt/vol), and 80- μ m thick slices were cut with a vibratome. Slices were incubated in 5% normal goat serum plus 0.3% Triton X-100 in saline for 2 hours at room temperature and, subsequently, in primary antibodies overnight at 4°C. After washing in 1% bovine serum albumin in saline, secondary antibodies were applied for 2 hours at room temperature. The lack of unspecific staining was proven by negative controls omitting the primary antibodies (not shown). Images were recorded with the confocal laser scanning microscope at single planes; excitation and emission settings were held constant for all images acquired. To label blood vessels and immune cells, retinal slices were exposed to Cy3-tagged *Griffonia simplicifolia* agglutinin isolectin IB₄. The isolectin labeled D-galactose residues located on endothelial cells and activated immune cells (microglial cells and blood-derived immune cells).

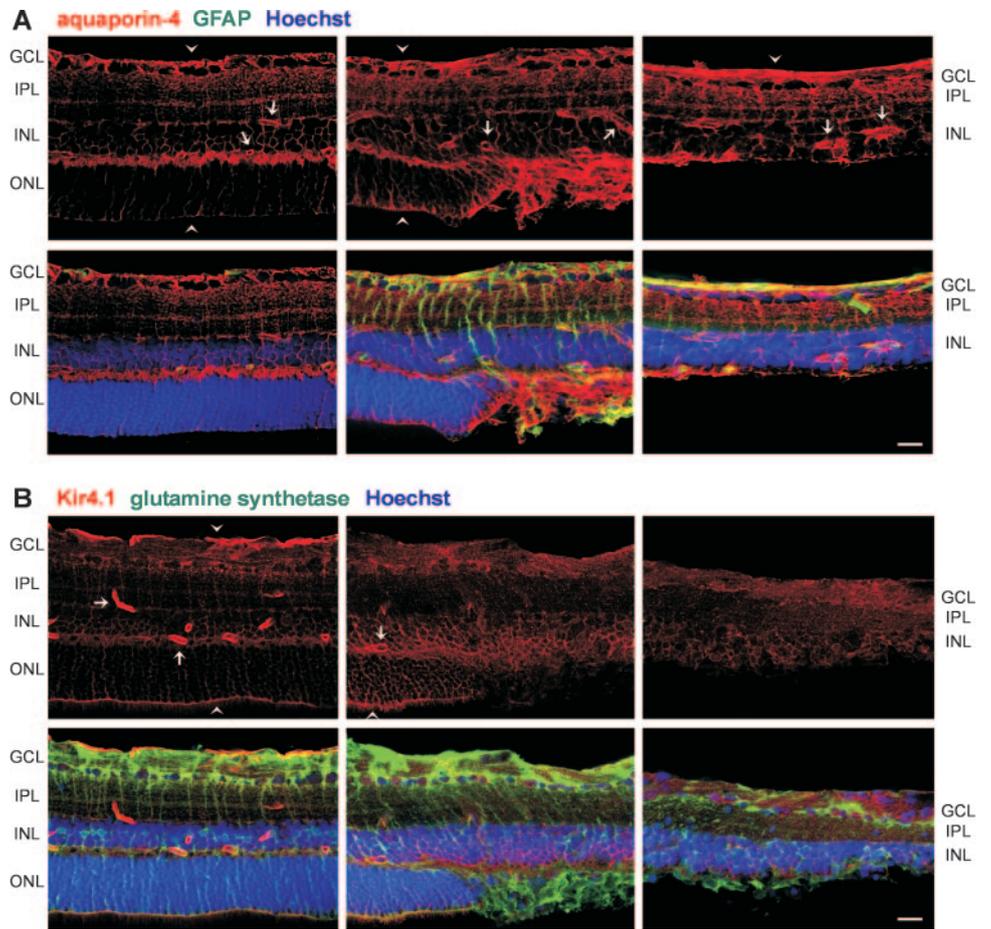


FIGURE 1. Treatment with blue light causes alterations in the immunolocalization of Kir4.1 and aquaporin-4 in the rat retina. Retinal slices were stained 3 days after light exposure. Injured retinal areas (*right*), uninjured areas (*left*), and transition zones (*middle*). (A) Staining of aquaporin-4 and the glial intermediate filament GFAP. (B) Colabeling of Kir4.1 and glutamine synthetase. Cell nuclei were labeled with Hoechst 33258 (blue). Arrows: perivascular labeling. Arrowheads: inner and outer limiting membranes. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars, 20 μ m.

Statistical Analysis

To determine the extent of soma swelling, the cross-sectional area of chloromethyltetramethylrosamine (Mitotracker Orange; Molecular Probes)–stained Müller cell bodies in the inner nuclear layer of retinal slices was measured offline using image analysis software of the laser scanning microscope. Bar diagrams displayed the mean cross-sectional areas of Müller cell somata that were measured after a 4-minute perfusion of the hypotonic solution, in percentages of the soma areas measured before osmotic challenge (100%). Amplitudes of the inward currents of isolated cells were measured at the end of the 250-ms voltage step from -80 to -140 mV. Statistical analysis was conducted with commercial software (SigmaPlot; SPSS Inc., Chicago, IL) and the Graphpad program (Prism; GraphPad, San Diego, CA); significance was determined by the Mann-Whitney *U* test for two groups, the Kruskal-Wallis test followed by Dunn comparison for multiple groups, and the Fisher exact test. Data are expressed as mean \pm SD (electrophysiological data) and mean \pm SEM (thickness of retinal layers, cell swelling data), respectively.

RESULTS

Retinal Degeneration after Blue Light Treatment

Treatment of rat retinas with blue light for 30 minutes resulted in an almost complete disappearance of photoreceptor cells in the irradiated areas within 3 days (Fig. 1, right column); few photoreceptor cell nuclei were found in the outer retina (see Fig. 3). Along with the degeneration of the outer retina, immunoreactivity for aquaporin-1 disappeared, and activated immune cells were found in the outer retina (Fig. 2), possibly representing activated retinal microglial cells or macrophages

invading the outer retina through the disrupted outer limiting membrane (Fig. 3). Degeneration of the photoreceptor cells was apparent 2 days after light treatment, as indicated histologically by the disaggregation of the outer nuclear and plexiform layers, the presence of cystoid spaces in the outer retina, and the disruption of the outer limiting membrane (Fig. 3). After 3 days of light treatment, a relatively sharp transition

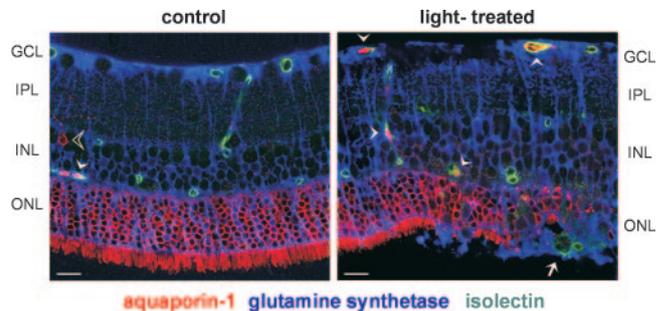


FIGURE 2. Blue light-evoked photoreceptor degeneration is associated with a disappearance of aquaporin-1 immunoreactivity in the outer retina of the rat. Slices of a control retina and a retina obtained 3 days after light treatment were immunostained against aquaporin-1 (red) and the Müller cell marker glutamine synthetase (blue). Blood vessels and activated immune cells were stained with isolectin (green). *Right*: transition zone between the injured and the uninjured tissue. *Arrow*: isolectin-stained immune cell (invading macrophage or activated microglia). *Filled arrowheads*: aquaporin-1 expressing erythrocytes. *Unfilled arrowhead*: aquaporin-1-positive amacrine cell. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars, 20 μ m.

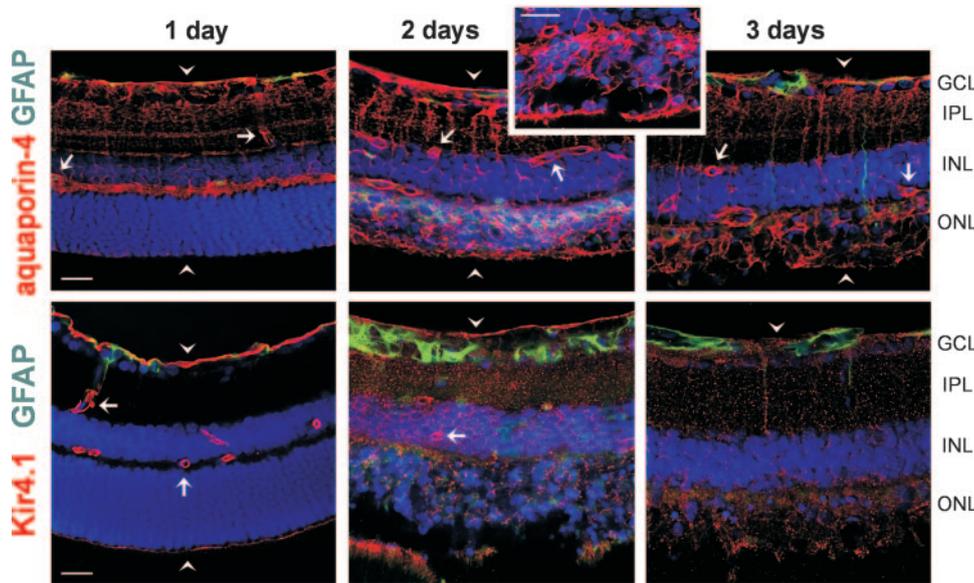


FIGURE 3. Time dependency of the alterations in Kir4.1 and aquaporin-4 labeling in the rat retina after treatment with blue light. Slices were immunostained 1, 2, and 3 days after light exposure. *Inset:* aquaporin-4 staining of the outer retina at higher magnification (2 days). Cell nuclei were labeled with Hoechst 33258 (blue). *Arrows:* perivascular labeling. *Arrowheads:* limiting membranes of the retina. Note the disruption of the outer limiting membrane in slices obtained 2 and 3 days after light exposure. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars, 20 μm .

between the light-damaged retinal areas and the surrounding uninjured areas was present (Fig. 1, middle column). In addition to the degeneration of the outer retina, there was also a degeneration of the inner retina, as indicated by the decreased thickness, particularly of the inner plexiform layer (Fig. 1). The thickness of the inner plexiform layer of light-exposed retinal areas was $30.4 \pm 1.7 \mu\text{m}$, which was significantly ($P < 0.01$) smaller than in the uninjured retinal periphery ($42.6 \pm 3.7 \mu\text{m}$; five animals).

Müller Cell Responses after Blue Light Treatment

In control retinas and in uninjured areas of light-treated retinas, immunoreactivity of the glial water channel, aquaporin-4, was predominantly located in the inner retina and in the outer plexiform layer, whereas staining of the outer nuclear layer (containing the photoreceptor cell bodies) was faint (Fig. 1A, left). In the inner retina, aquaporin-4 was concentrated around the vessels and at the inner limiting membrane. Light treatment caused a time-dependent increase in aquaporin-4 immunoreactivity in the outer nuclear layer that was apparent 2 days after treatment, whereas perivascular localization in the inner retina remained largely unaltered (Fig. 3). After 3 days, the edges of the light-exposed areas (Fig. 1A, middle) and the spaces around the few remaining photoreceptor cells (Fig. 3) displayed increased aquaporin-4 in the outer retina. Müller cells undergoing reactive gliosis increased the expression of GFAP immunoreactivity.¹⁴ Although this increase in GFAP was also observed in light-injured areas, it was particularly prominent in the transition zone between the injured and uninjured areas (Fig. 1A). The presence of GFAP (Fig. 1A) and glutamine synthetase in the outer retina (Fig. 1B) suggested that aquaporin-4 was expressed by reactive Müller cells.

Exposure to blue light caused a dislocation and a decrease of Kir4.1 protein in the retina. In uninjured retinal tissues, Kir4.1 was concentrated around the vessels and at the limiting membranes (Fig. 1B, left). In injured areas, Kir4.1 was redistributed from these prominent expression sites and displayed a more or less even distribution, at low level, throughout the entire retinal tissue (Fig. 1B, right). At the borders of the light-damaged areas, there was a transition between the two staining patterns (Fig. 1B, middle). A more even distribution of the Kir4.1 immunostaining was observed up to 120 to 160 μm laterally from the borders of the injured retinal areas. An alteration in the retinal distribution of Kir4.1 immunoreactivity was

first observed 2 days after light treatment, whereas prominent staining of the perivascular membranes and at the inner limiting membrane disappeared after 3 days (Fig. 3).

After blue light treatment, Müller cells displayed a strong time-dependent decrease in potassium conductance (Fig. 4A). The inward potassium currents decreased to $70.1\% \pm 40.4\%$ of control (100%) 2 days after light treatment ($P < 0.05$) and to $13.8\% \pm 10.7\%$ of control after 3 days ($P < 0.001$) (Fig. 4B). (Similar results were obtained when the potassium currents were recorded at 35°C to 37°C . Three days after light treatment, the amplitude of the inward potassium currents of cells from light-treated retinal areas was $617 \pm 561 \text{ pA}$ [$n = 5$], which was significantly different from the value obtained in cells from control tissues, $3109 \pm 609 \text{ pA}$ [$n = 5$; $P < 0.01$].) The decrease in glial potassium conductance was observed only in cells isolated from injured areas of the light-treated retinas, not in cells from the uninjured retinal areas (Fig. 4B). This difference corresponded well with the alteration in the retinal distribution of Kir4.1, which was observed only in the injured areas (Fig. 1B). Because Kir4.1 is the main channel subtype that contributes to the potassium conductance of Müller cells,¹⁶ the data suggest that the redistribution of the Kir4.1 protein is accompanied by functional inactivation of the channels. Kir4.1 channels are the major determinant of the high resting membrane potential of Müller cells.¹⁶ Müller cells isolated from injured retinal areas 3 days after light treatment displayed a significant ($P < 0.001$) membrane depolarization (to $-54.6 \pm 19.3 \text{ mV}$) compared with cells from untreated control retinas ($-87.1 \pm 5.2 \text{ mV}$) or cells from uninjured retinal areas of the treated eyes ($-86.0 \pm 5.1 \text{ mV}$; Fig. 4C). A decrease in Kir channel-mediated potassium currents of rat Müller cells during retinopathy is often associated with the emergence of fast transient (A-type), outwardly rectifying potassium currents; this alteration was observed, for example, in Müller cells during ocular inflammation and retinal ischemia-reperfusion.^{18,19} We found that none of the Müller cells investigated from untreated control retinas or from noninjured retinal areas of the light-treated eyes displayed A-type potassium currents (Fig. 4D); in these cells, the outward potassium conductance (recorded during blockade of the Kir channels with 100 μM barium chloride) was dominated by slowly activating delayed rectifier currents (not shown). However, 69% and 100%, respectively, of Müller cells isolated from injured retinal areas 2 and 3 days after light treatment displayed A-type potas-

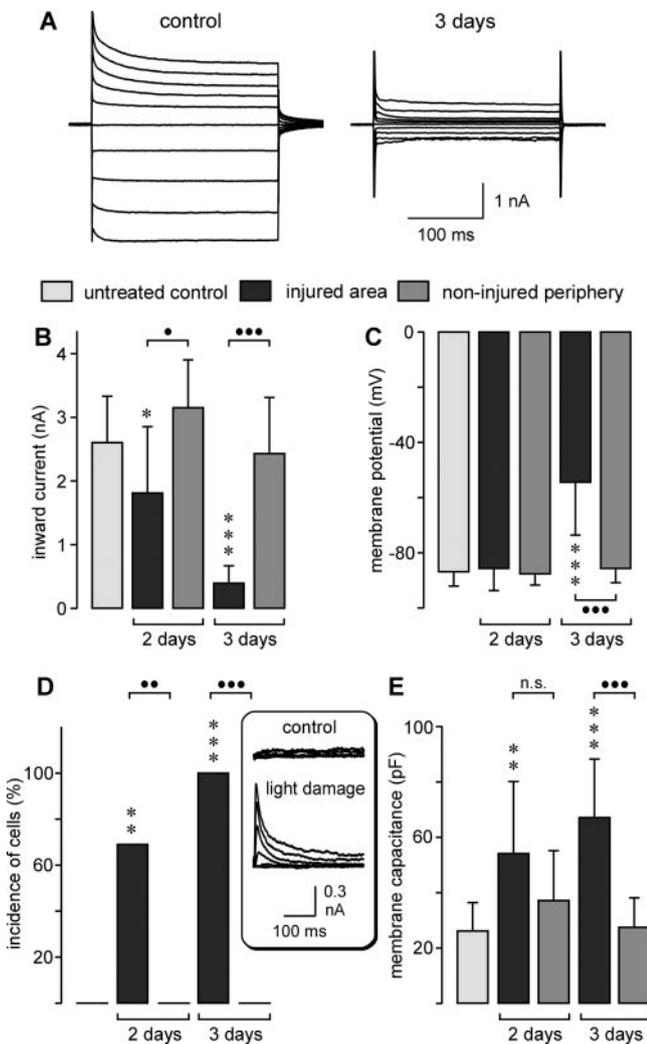


FIGURE 4. Treatment of rat retinas with blue light alters the potassium conductance of Müller glial cells. Data were obtained 2 and 3 days after light exposure. Recordings were made at room temperature. (A) Representative traces of whole-cell currents in cells isolated from an untreated control retina and a light-treated retina. Potassium currents were evoked by 20-mV incremental voltage steps between -160 and $+20$ mV from a holding potential of -80 mV. Outward currents are depicted upwardly, and inward currents are depicted downwardly. (B) Mean amplitude of the inward potassium currents in cells isolated from untreated control eyes and from light-damaged areas and the noninjured periphery of the treated retinas. (C) Resting membrane potential. (D) Incidence of cells that displayed fast transient A-type potassium currents. *Right:* representative traces show the absence of A-type potassium currents in a cell from a control retina and the presence of such currents in a cell from a light-damaged retina. Currents were isolated by the difference protocol described in Materials and Methods, in the presence of the Kir channel blocker barium chloride ($100 \mu\text{M}$). (E) Cell membrane capacitance. Each bar represents values obtained in 10 to 21 cells isolated from four (2 days) and five (3 days) animals, respectively. Significant differences versus untreated control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant differences between injured and uninjured areas of the treated retinas: ● $P < 0.05$; ●● $P < 0.01$; ●●● $P < 0.001$. n.s., not significant.

sium currents on membrane depolarization (Fig. 4D). The emergence of A-type potassium currents correlated to the decrease in the Kir channel-mediated potassium currents after light treatment (Fig. 4B). Moreover, we found that Müller cells of injured retinal areas displayed a significant increase in plasma membrane area (as indicated by the increase in the cell

membrane capacitance; Fig. 4E), suggesting hypertrophy of Müller cells in response to light-evoked photoreceptor degeneration. Again, such an increase in the cell membrane area was not found in cells isolated from uninjured areas of the treated retinas (Fig. 4E).

Another alteration of Müller cells that has been associated with a decrease in functional Kir4.1 channels is the induction of cellular swelling in response to hypo-osmotic stress.^{17,18} A similar alteration in the osmotic swelling properties of Müller cells was found after treatment of rat retinas with blue light. Perfusion of slices of light-injured retinas with a hypo-osmolar solution (which contained 60% of control osmolarity) caused a significant ($P < 0.001$) increase in the size of the Müller cell somata by approximately 15% (Figs. 5A, 5B). Such swelling was observed at room temperature (Fig. 5B) and at 35°C to 37°C (Fig. 5C). On the other hand, Müller cells in slices of untreated control retinas did not significantly alter somata sizes under these conditions (Fig. 5B). Similarly, Müller cell bodies in slices of the noninjured periphery of treated retinas did not swell significantly under hypo-osmotic conditions. Blockade of the Kir channels by barium ions, however, caused swelling of Müller cell bodies in response to hypo-osmotic stress in all Müller cell populations investigated (Fig. 5B). The data indicated that treatment of rat retinas with blue light caused an alteration in the osmotic swelling properties of Müller cells reflected by a loss of their ability to efficiently regulate cellular volume under varying osmotic conditions.

In addition to a blockade of Kir channel-mediated potassium currents, inflammatory mediators, such as arachidonic acid and prostaglandins, and oxidative stress have been causally implicated in the induction of osmotic Müller cell swelling.^{26,27} Application of arachidonic acid, prostaglandin E_2 , or hydrogen peroxide caused a swelling of Müller cell bodies in slices of untreated control retinas under hypo-osmotic conditions (Fig. 6A). The swelling of Müller cell bodies in control retinas evoked by hypo-osmolar solution containing barium chloride was prevented when the formation of arachidonic acid was blocked by preincubation of the slices with a selective inhibitor of the phospholipase A_2 (PLA_2), 4-bromophenacyl bromide (Fig. 6B). Similarly, swelling was inhibited in the presence of an inhibitor of the cyclooxygenase indomethacin (Fig. 6B). The anti-inflammatory glucocorticoid triamcinolone acetonide is used clinically for the rapid resolution of retinal edema. Osmotic swelling of Müller cell bodies in control retinas was fully prevented in the presence of triamcinolone in the hypotonic bathing solution (Fig. 6B). The inhibitory effect of the cell-permeable, dithiol-reducing agent dithiothreitol suggested that acute oxidative stress plays a causative role in the swelling of Müller cells (Fig. 6B). All these agents also blocked the swelling of Müller cell bodies in blue light-treated retinas (Fig. 6C). The data suggest an involvement of acute oxidative stress and an endogenous formation of arachidonic acid and prostaglandins in evoking Müller cell swelling under hypo-osmotic conditions and may indicate an alteration in the functional state or expression of inflammatory enzymes in Müller cells in light-treated retinas.

DISCUSSION

Exposure of the sensory retina to excessive light results in photoreceptor cell death and damage to the pigment epithelium; both degenerative events contribute to the development of local edema in the outer retina. However, excessive light also causes degenerative alterations in the inner retina, such as apoptotic death of ganglion cells.^{10,11} Given that Müller cells maintain the osmohomeostasis of the inner retina, we were interested in investigating whether these cells respond to

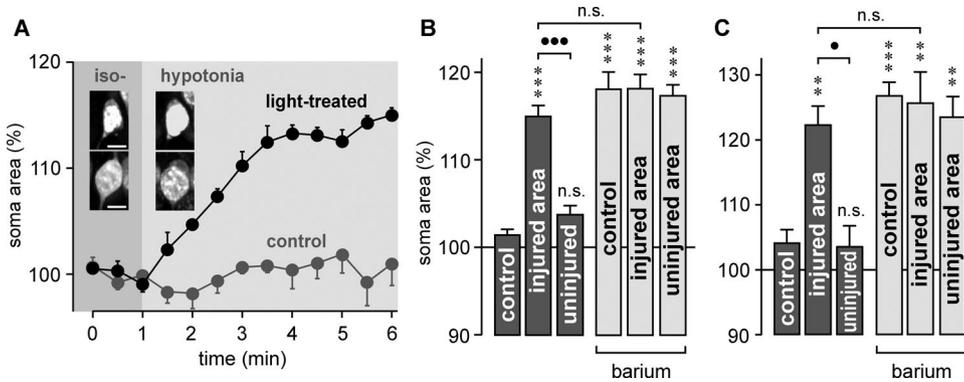


FIGURE 5. Treatment of rat retinas with blue light causes an alteration in the osmotic swelling properties of Müller glial cells. Data were obtained from untreated (control) retinas and from retinas isolated 3 days after light exposure. (A) Exposure to a hypo-osmolar solution (60% of normal osmolarity) induced time-dependent swelling of Müller cell bodies in slices from a light-injured retina and had no effect on the size of Müller cell bodies in an untreated (control) retinal slice. Data are mean cross-sectional areas of five cell bodies. Images display original records of dye-filled Müller cell bodies in a slice of a

light-injured retina, recorded before (*left*) and during (*right*) hypo-osmotic exposure. Scale bars, 5 μ m. (B, C) Mean cross-sectional area of Müller cell bodies after 4 minutes of hypo-osmotic exposure, in the absence and presence of barium chloride (1 mM). Cells were recorded in slices of untreated (control) retinas and light-injured retinas and in slices of the noninjured periphery of treated retinas. Data were obtained at 22°C to 25°C (B) and 35°C to 37°C (C), respectively, and are expressed in percentages of the values measured before stimulation (100%). Each bar represents values obtained in 7 to 50 cells in slices from 3 to 5 animals. Significant differences versus untreated control: ** $P < 0.01$; *** $P < 0.001$; ● $P < 0.05$; ●●● $P < 0.001$. n.s., not significant.

edema in the outer retina and whether excessive light evokes gliotic changes of Müller cells that may contribute to degenerative alterations in the inner retina.

Müller Cell Responses to Light-Induced Retinal Degeneration

Retinal light damage evokes a reactivation of Müller cells that is characterized by, for example, cellular hypertrophy, increased immunoreactive GFAP, decreased expression of the glutamine

synthetase, and increased expression of distinct receptors and neurotrophic factors.^{28–34} Here, we confirm previous results that showed hypertrophy of Müller cells and increased immunoreactive GFAP in light-induced retinopathy. Blue light treatment of the rat retina induced an increase in the immunoreactivity of the glial water channel aquaporin-4 in the outer retina. This upregulation appears to be (part of) an adaptive response of the glial cells, enabling them to rapid clearance of extracellular water (or fast water supply, depending on the osmotic deviation). Blue light caused a decrease in the prominent Kir4.1 expression and dislocation of the Kir4.1 protein within the whole retinal tissue. This was accompanied by a strong decrease in the potassium conductance of Müller cells, suggesting functional inactivation of Kir channels. Despite the upregulation of aquaporin-4 in the outer retina, water fluxes through Müller cell membranes must be impaired when they cannot be balanced by accompanying ion fluxes.^{17,35} Indeed, we observed a conspicuous alteration in the osmotic swelling characteristics of Müller cells after the treatment of rat retinas with blue light. In the presence of a hypo-osmotic environment, Müller cells in slices of blue light-treated retinas displayed cellular swelling, whereas Müller cells in control retinas did not alter the volume of their somata. Müller cell swelling reflects an alteration of the transmembrane water transport in response to varying osmotic conditions. Downregulation of functional Kir channels has been causally related to the induction of osmotic Müller cell swelling,^{17,35} which is also suggested by the observation that a blockade of Kir channels by barium ions induces cellular swelling under hypo-osmotic conditions (Fig. 6B). It has been suggested that a rapid outflow of potassium ions through weakly rectifying Kir4.1 channels normally compensates the transmembrane osmotic gradient and, thus, prevents water influx and cellular swelling under hypo-osmotic conditions.¹⁷ Osmotic disturbances may occur in the retina in situ, resulting from an apoptosis-associated increase in the extracellular potassium and water content and an impaired clearance capability of Müller cells.

Possible Causes of Müller Cell Gliosis/Swelling

The current pattern of Müller cells in blue light-treated retinas, displaying low Kir currents and a high expression level of A-type potassium currents, resembles the pattern found in developing Müller cells in the young postnatal retina, before differentiation to mature cells.³⁵ Similarly, an even distribution of Kir4.1 protein along the Müller cell membrane (with the loss

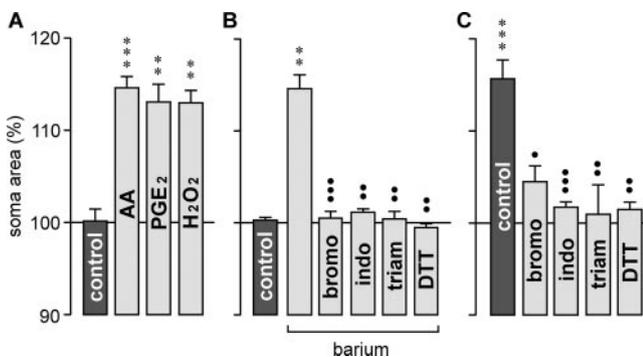


FIGURE 6. Osmotic swelling of Müller cell bodies is mediated by activation of inflammatory enzymes and oxidative stress. Data are mean (\pm SEM) cross-sectional areas of Müller cell bodies obtained from untreated retinas (A, B) and from retinas isolated 3 days after light exposure (C). Values were measured at room temperature after 4-minute perfusion of retinal slices with a hypo-osmolar solution (control; 60% of normal osmolarity) and are expressed in percentages of the values measured before stimulation (100%). (A) Perfusion of retinal slices from untreated eyes with a hypo-osmolar solution containing arachidonic acid (AA; 10 μ M), prostaglandin E₂ (PGE₂; 30 nM), and hydrogen peroxide (H₂O₂; 50 μ M), respectively resulted in a swelling of Müller cell bodies. (B) Swelling of Müller cell bodies in slices of untreated retinas under hypo-osmotic conditions in the presence of barium chloride (1 mM) was prevented by the following agents: the selective inhibitor of PLA₂ activation 4-bromophenacyl bromide (bromo; 300 μ M), the cyclooxygenase inhibitor indomethacin (indo; 10 μ M), the corticosteroid triamcinolone acetonide (triam; 100 μ M), and the reducing agent dithiothreitol (DTT; 3 mM), respectively. (C) Swelling of Müller cell bodies in slices of light-injured retinas was inhibited by the respective agents. Each bar represents values obtained in 8 to 10 cells. Significant differences versus untreated control: ** $P < 0.01$; *** $P < 0.001$. Significant blocking effects: ● $P < 0.05$; ●● $P < 0.01$; ●●● $P < 0.001$.

of the prominent localization in perivascular membranes and at the limiting membranes of the retina) has been described as an early postnatal pattern of Kir4.1 expression.³⁵ Together these findings suggest a distinct dedifferentiation of Müller cells after exposure to blue light. A similar dedifferentiation of Müller cells has been described in animal models of retinal ischemia-reperfusion and ocular inflammation.¹⁷⁻¹⁹ It is known that light exposure evokes an inflammatory response in the retina and that inflammation represents part of the causal chain of retinal light damage.⁵ Excessive light triggers the release of proinflammatory cytokines and polyunsaturated fatty acids such as arachidonic acid.^{3,23,24} Another contributing factor to light-evoked photoreceptor cell death is oxidative stress induced by a prolonged increase in oxygen tension and photooxidation.^{21,22} Blue light is absorbed by various light-sensitive enzymes and proteins, such as by prostaglandin synthetases; the light-induced activation of these enzymes leads to the generation of inflammatory mediators and reactive oxygen radicals.³⁶ Reactive oxygen radicals may also be formed in the mitochondria after absorption of blue light by cytochrome oxidase.⁴ Other sources of reactive oxygen radicals and inflammatory factors are activated microglial cells and macrophages that phagocytose the debris of photoreceptor and pigment epithelial cells. Macrophages invade the degenerating outer retina and come in direct contact with Müller cells. Based on the present data, we cannot decide whether the initial damage in our model of retinal blue light injury was located in the photoreceptors (class 1 photochemical damage) or in the pigment epithelium (class 2 damage).^{37,38} Although it is likely that several types of blue light-induced photochemical retinal damage may coexist,⁴ it has been shown that short-wavelength, high-intensity light initially evokes class 2 damage, whereas long-term, low-intensity white light initially damages the photoreceptors (for a review, see Wu et al.⁴). The preservation of the outer retinal histologic structure 1 day after blue light treatment (Fig. 3) may suggest initial damage of the pigment epithelium in our model. However, determination of the cascade of injury in our model needs further investigation.

The involvement of inflammatory and oxidative stress conditions in the response of Müller cells to blue light is further supported by the ameliorating effects of antioxidants and blockers of inflammatory mediators onto Müller cell swelling (Fig. 6). The osmotic swelling of Müller cells was prevented when the activity of the phospholipase A₂ or the cyclooxygenase was pharmacologically blocked, suggesting the involvement of arachidonic acid and prostaglandins. In addition, the inhibition of oxidative stress by application of a reducing agent prevented the swelling of Müller cells. It is known that oxidative stress can initiate lipid peroxidation, resulting in the release of arachidonic acid from membrane phospholipids,^{39,40} and that the activity of the phospholipase A₂ is increased in response to osmotic challenge and oxidative stress, resulting in peroxidation of membrane phospholipids.^{41,42} Free radicals and hydroperoxides stimulate the activities of lipoxygenase and cyclooxygenase.⁴³ Arachidonic acid and prostaglandins potently inhibit the sodium pump activity that leads to intracellular sodium overload and cellular swelling.^{44,45} Furthermore, arachidonic acid blocks membrane channels, such as volume-regulated anion and outwardly rectifying potassium channels involved in the extrusion of osmolytes.^{46,47} On the other hand, Müller cells in control retinas are able to release potassium through Kir4.1 channels that balances osmotic gradients across the plasma membrane, thus preventing the activation of inflammatory enzymes in response to osmotic challenge. Very likely, the observed increase in aquaporin-4 in light-damaged retinas (Fig. 1) further contributes to the osmotic swelling of Müller cells. Aquaporin-4 mediates a high water permeability of glial membranes⁴⁸; an increase in aqua-

porin-4 expression is associated with a swelling of cultured brain astrocytes.⁴⁹ However, further investigations are necessary to determine the intracellular signaling involved in Müller cell swelling.

Possible Contribution of Müller Cells to Inner Retinal Degeneration

Treatment with blue light induces (in addition to photoreceptor degeneration) degeneration of the inner retina, as suggested by the decrease in the thickness of the inner plexiform layer (Fig. 1). A preferential decrease in the thickness of the inner plexiform layer represents a typical degeneration pattern of the rodent retina early after transient ischemia.⁵⁰ Retinal ischemia-reperfusion injury is, at least in part, mediated by the toxicity of excess glutamate and by damaging oxygen-derived free radicals formed during reperfusion.⁵¹ Dislocation and functional inactivation of Kir4.1 may accelerate degeneration of the inner retina. Potassium currents through Kir4.1 channels are thought to be crucial in the spatial buffering of imbalances in the extracellular potassium concentration caused by neuronal activity.¹⁶ Inactivation of Kir4.1 impairs potassium clearance by Müller cells, resulting in neuronal hyperexcitation and glutamate toxicity. Similar redistribution of the Kir4.1 protein has been described in retinas of mice with genetic inactivation of the dystrophin gene product Dp71,⁵² which is proposed to be involved in the anchoring and clustering of Kir4.1 channels in glial membranes.⁵³ The altered distribution of Kir4.1 protein in Dp71-null mice was accompanied by an enhanced vulnerability of retinal ganglion cells to ischemia-reperfusion injury.⁵² Inflammation is a major pathogenic factor of retinal edema,⁵⁴ which is suggested to be mediated in part by swelling of Müller glial cells after the downregulation/inactivation of Kir4.1 channels.²⁰ Retinal edema may be a consequence of, and may contribute to, neuronal degeneration by direct mechanical damage, compression of blood vessels, and other mechanisms, including a decrease in the extracellular space volume that favors neuronal hyperexcitation. The present results suggest a contribution of Müller cells to inner retinal degeneration after blue light injury of the retina; however, one should keep in mind that reactive Müller cells may contribute to neuronal survival in the light-damaged retina by the expression of neurotrophic factors.^{31,34}

SUMMARY AND CONCLUSIONS

In summary, we found that Müller cells respond to excessive blue light with alterations in the localization of Kir4.1 and aquaporin-4 channel proteins. Upregulation of aquaporin-4 may support the resolution of edema in the outer retina. Dislocation of the Kir4.1 protein is accompanied by a functional inactivation of the Kir channels that results in impairment of retinal potassium homeostasis. The functional inactivation of Kir4.1 channels also alters transmembrane water transport, promoting Müller cell swelling under varying osmotic conditions. Impairment of the potassium and water homeostasis caused by functional inactivation of Kir4.1 channels in Müller cells may contribute to neuronal hyperexcitation and excitotoxicity in the inner retina. Thus, gliotic alterations of Müller cells may represent one causative factor contributing to the degenerative changes in the inner retinal layers observed after excessive light exposure. Our results provide new insight into the devastating effects of blue light in the retina and may argue in favor of antioxidative and anti-inflammatory treatment/prevention of injuries such as iatrogenic macular edema and age-dependent macula degeneration.

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