Aquaporin-4 Gene Disruption in Mice Protects against Impaired Retinal Function and Cell Death after Ischemia

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PURPOSE. Water channel aquaporin (AQP-4) is expressed in Müller cells in retina, which are similar to astroglial cells in the central nervous system, where AQP4 deletion protects against cytotoxic brain edema after cerebral ischemia. A transient ischemia-reperfusion model was used to determine whether AQP4 deletion in mice protects the retina.

METHODS. Retinal function and morphology were assessed in wild-type versus AQP4-deficient mice after ischemic damage produced by a 45- to 60-minute elevation of intraocular pressure to 120 mm Hg. Retinal function was assessed by electroretinography, and retinal structure by light microscopy. Extracellular space (ECS) size in fluorescently stained retinal slices was assessed by fluorescence recovery after photobleaching.

RESULTS. Retinal function and cell survival were significantly improved in AQP4-deficient mice in both inbred (C57/b6j) and outbred (C1D1) genetic backgrounds. By electoretinography, b-wave amplitude was reduced by 75% to 83% at 1 to 4 days after ischemia in wild-type mice versus 48% to 51% in AQP4-null CD1 mice. Reductions were 53% to 72% versus <34% in C57/b6j mice. Retinal structure and cell count were preserved in AQP4-null mice, particularly in the inner nuclear and plexiform layers of the retina, where Müller cells are concentrated. At 4 days after ischemia, inner retinal thickness was thinned by 43% in wild-type mice versus 11% in AQP4-null mice. Several mechanisms for retinal protection were investigated, including ECS expansion, reduced early swelling, and altered Kir4.1 K+ channel expression.

CONCLUSIONS. AQP4 deletion in mice is neuroprotective in a transient ischemia model of retinal injury, suggesting the possible use of AQP4 inhibitors in retinal vascular occlusive and ischemic diseases. (Invest Ophthalmol Vis Sci. 2004;45:4477–4483) DOI:10.1167/iovs.04-0940

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performed in matched wild-type and knockout mice of ages 8 to 10 weeks in CD1 and C57/Bl6 genetic backgrounds. Mice were housed in the University of California at San Francisco animal facility, fed standard mouse chow (4% fat), and maintained in a 12-hour light–dark cycle. In all studies, the investigators were blinded to genotype information until completion of the analysis. Protocols were approved by the University of California San Francisco Committee on Animal Research and are in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Ischemia Model

After anesthesia with intraperitoneal ketamine (80 mg/kg) and xylazine (16 mg/kg), pupils were dilated with 1% tropicamide and 2.5% phenylephrine applied topically. Retinal ischemia in one eye was produced by increasing intraocular pressure to 120 mm Hg for 45 or 60 minutes by introducing into the anterior chamber a micropipette containing an isotonic salt solution (Cytosol Ophthalmics, Inc., Braintree, MA) connected to a reservoir situated at an appropriate height. Retinal ischemia was verified by whitening of the anterior segment of the globe and blanching of episcleral veins, as viewed by stereo light microscopy. Body temperature was maintained at 37 ± 0.5°C with a heating pad. The opposite eye served as the nonischemia control. At the end of the period of ischemia, the micropipette was removed and 0.3% tobramycin ointment (Alcon, Fort Worth, TX) was applied to the conjunctival sac.

Retinal Morphology and Immunocytochemistry

After mice were killed by cervical dislocation, eyes were enucleated and frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA) on dry ice. Cryostat sections were cut sagittally through the optic nerve at 7 μm thickness and fixed in 4% paraformaldehyde in PBS for staining by hematoxylin and eosin and immunocytochemistry. Thicknesses of the inner nuclear layer (INL) and the inner plexiform layer (IPL) were measured, as well as the internal retinal layer (IRL), bounded by the internal limiting membrane (ILM) and the interface between the ONL and outer plexiform layer (OPL), and the outer retinal layer (ORL), bounded by the RRE layer and the interface between the ONL and OPL. Layer thicknesses were measured in three cryostat sections per retina of at least three mice at a distance of 300 μm from the optic nerve. The number of cells in the INL and ganglion cell layer (GCL) were counted over a 100-μm length at a distance of 300 μm from the optic nerve. Immunocytochemistry was performed with a polyclonal anti-AQP4 antibody raised against the AQP4 C terminus (Chemicon, Temecula, CA) or an anti-Kir4.1 antibody (Alomone Laboratories, Jerusalem, Israel), with Cy3-coupled secondary antibody (Sigma-Aldrich, St. Louis, MO). Retinal sections were photographed with a fluorescence microscope (model D85000; Leica Microsystems, Bannockburn, IL).

Electroretinography

ERG measurements were performed as described previously.52 Full-field retinal illumination was accomplished by focusing the output of a strobe flashlamp (NovaStrobe-Stroboscope; Monarch Instruments, Anhersert, NH) onto a fiber-optic bundle delivering light to the retina via a Lucite (DuPont, Wilmington, DE) coupler that conformed to the curvature of the globe. The coupler made contact with the globe using methylcellulose solution (Gonisol; Iolab Pharmaceuticals, Akron, OH). Flash intensities (white light, 20-methylcellulose solution (Gonisol; Iolab Pharmaceuticals, Akron, OH)). Retinal Morphology and Immunocytochemistry was verified by whitening of the anterior segment of the globe and blanching of episcleral veins, as viewed by stereo light microscopy. Body temperature was maintained at 37 ± 0.5°C with a heating pad. The opposite eye served as the nonischemia control. At the end of the period of ischemia, the micropipette was removed and 0.3% tobramycin ointment (Alcon, Fort Worth, TX) was applied to the conjunctival sac.

Fluorescence Recovery after Photobleaching of Retinal Slices

A fluorescence recovery after photobleaching (FRAP) method developed previously59 was adapted to assess the ECS in retinal slices. After mice were killed by cervical dislocation, one eye was enucleated, placed in oxygenated artificial cerebrospinal fluid (aCSF, in mM: NaCl, 124; KCl, 4; MgCl2, 1; CaCl2, 2.5; KH2PO4, 1; and glucose, 10; pH 7.4), and bubbled with 95% O2 and 5% CO2 at 4°C. After excision of the cornea, the whole retina was carefully separated within 3 to 5 minutes from the lens, vitreous body, and wall of the globe. The retina was then embedded in 2% low-melting-point agarose, and retinal slices of 200-μm thickness were cut (Vibratome; World Precision Instruments, Sarasota, FL) in oxygenated aCSF at 4°C. Slices were placed on a semipermeable filter insert (0.4-μm pore size; Corning Costar, Corning, NY), and the ECS was fluorescently stained by incubation for 15 minutes at 4°C in aCSF containing FITC-dextran (4 kDa, 50 mg/mL; Sigma-Aldrich). Excess dye was washed with aCSF, and the slice was covered with mineral oil. The filter insert containing the fluorescently stained retinal slice was then placed in a 35°C incubator (Harvard Apparatus, Holliston, MA) on the stage of an upright epifluorescence microscope (Nikon, Melville, NY) in which the undersurface of the porous insert was perfused with oxygenated aCSF.

The INL and ONL were identified in retinal slices by transmitted light microscopy. Light from an Argon ion laser (1–2 mW, 488 nm) was focused onto the INL or ONL in the retinal slice with a 50× air-objective lens (numerical aperture 0.55; Nikon), and emitted fluorescence was detected by a gated photomultiplier after passing through a 510-nm dichroic mirror and 510-nm long-pass filter. Confoicality was achieved by positioning a 400-μm pinhole at the back focal plane in the detection path. Photobleaching was accomplished by increasing laser illumination by 2500- to 5000-fold briefly (0.5–2 ms) using an acousto-optic modulator. Control studies were performed to assure that there was no bleaching by the dim beam probe. Fluorescence recovery curves, R(t), were fitted using the semiempirical equation: 

\[
R(t) = R_0 + \left[ (R(t) - R_0) \right] \left[ 1 + (t/\tau) \right]^{-1},
\]

where \( R_0 \) is the fluorescence immediately after bleaching, \( R \) is the mobile fraction, and \( \tau \) is the recovery half-time.40 Data from at least three different spots in the ONL and INL were averaged for each retina. Relative FITC-dextran diffusion in retina versus saline (\( D/D_0 \)) was determined from \( \tau \) measured in a solution standard (50 mg/mL FITC-dextran in PBS): 

\[
D/D_0 = \tau^{(0)}/\tau^{(s)}.
\]

RESULTS

Figure 1A shows AQP4 immunostaining in the retina of a wild-type (+/+) mouse, with localization to the vitreal surface and surrounding vessels in the internal retina, which correspond to Müller cell end feet as found in rat and human eye.33 Specific immunostaining was absent in the retina of AQP4-null (−/−) mice. Kir4.1 immunostaining was similar in the wild-type and AQP4-null mice, with localization to membrane regions facing the vitreous body and around vessels, as reported previously.53

Ischemic retinal damage was produced by transient elevation of intraocular pressure to 120 mm Hg to abolish blood flow, as diagrammed in Figure 1B. A glass microneedle was inserted into the anterior chamber through the cornea and connected to a fluid reservoir to set intraocular pressure. Figure 1C shows representative light micrographs of retina before
and at indicated times after 60 minutes of transient ischemia. The highly cellular INL and ONL are visible, with the inner surface of the retina containing ganglion cells marked by an asterisk. Initial swelling of internal retinal layers was noted at 6 to 12 hours after ischemia, with subsequent thinning of the retina and marked loss of cellularity in the INL and GCL, and thinning of the IPL (between the INL and GCL).

The baseline ERG response of wild-type and AQP4-null mice aged 8 to 10 weeks, as used in subsequent retinal ischemia studies, was established. Figure 2A shows representative flash ERG recordings in the two mouse strains and genotypes. ERG waveforms are shown over a 5 order-of-magnitude range of flash intensities. At the higher light intensities the ERG consisted of an initial downward deflection (a-wave) followed by a slower transient current elevation (b-wave) with oscillatory potentials (OPs) observed in the early upward portion of the b-wave. The OPs at the highest flash intensity are shown on an expanded scale (after high-pass filtering) at the bottom. Qualitatively, there was little effect of AQP4 deletion on ERG waveforms in CD1 mice, but a significant reduction in b-wave amplitudes in C57/bl6 mice. Averaged data are summarized in Figure 2B, showing reduced b-wave amplitudes in AQP4-deficient C57/bl6 mice at all flash intensities, without significant change in a-wave amplitudes. The b-wave is thought to arise from the bipolar cell depolarization accompanying Müller cell activation and light-evoked increase in ECS [K⁺], whereas the a-wave is produced by photoreceptor cell activation. In addition, the amplitude of OPs was reduced significantly in AQP4-deficient C57/bl6 mice (from 4.4 ± 0.2 to 3.1 ± 0.2 μV; P < 0.05) at the highest flash intensity. Subsequent ischemia studies were performed on both mouse strains to ensure the robustness of results in inbred versus outbred genetic backgrounds and in baseline differences in retinal responses.

ERG analysis was performed on a series of mice before and at 1, 2, and 4 days after 45 minutes of transient retinal ischemia. For these functional measurements, 45 minutes of ischemia was used because initial experiments showed marked reductions in evoked potentials with 60 minutes of ischemia. ERG waveforms in Figure 3A show relative preservation of b-wave and OP amplitudes in the AQP4-null mice. Averaged ERG waveform amplitudes from all mice are summarized in Figure 3B. Analysis of absolute amplitudes for the highest flash intensity (Fig. 3B, left panels) revealed that the ischemia-induced reductions in b-wave and OP amplitudes were reduced in wild-type (filled circles) compared with AQP4-null (open circles) mice, without differences in reduction of a-wave amplitude. A paired analysis (Fig. 3B, right panels) was also performed, in which data were plotted as percentages of preischemia amplitudes computed for each mouse. The paired analysis shows significant preservation of b-wave and OP amplitudes after ischemic retinal injury in AQP4-null mice of both genetic backgrounds.

Retinal morphology was studied at different times after transient ischemia in wild-type versus AQP4-null mice. Representative sections in Figure 4A show similar retinal morphologies in wild-type versus AQP4-null mice before ischemia, with significant preservation of retinal thickness and cellularity from AQP4-null mice at 96 hours after ischemia, particularly in the internal retinal layers. Cell nuclei were counted in the ONL, INL, and GCL before and at 96 hours after 60 minutes of ischemia (Fig. 4B). The number of cells was similar in retinas from wild-type and AQP4-null mice under basal conditions; however, at 96 hours after ischemia there was significantly reduced cell loss in INL and GCL of retinas from AQP4-null mice (Fig. 4B). Thickness measurements of the outer versus inner retinal layers (IRL versus ORL) and of the INL and IPL before ischemia and at indicated times after 45 or 60 minutes of ischemia are summarized in Figure 4C. Retinal ischemia produced a marked reduction in IRL thickness especially in the INL and IPL at 96 hours after 45 or 60 minutes of ischemia, which was significantly greater in wild-type than in AQP4-null mice. Retinal swelling was observed in the IPL at 6 to 12 hours...
Slices. After embedding retinal tissue in agarose, we cut 200-
photobleaching was performed on fluorescently stained retinal
amplitudes. * 

Left genetic backgrounds. 

FIGURE 3. Electrotetretinography after retinal ischemia. (A) Representative ERGs before and at 1, 2, and 4 days after 45 minutes of retinal ischemia in wild-type and AQP4-null mice in CD1 (left) and C57/bl6 (right) genetic backgrounds. Data shown for highest light intensity. (B) Averaged waveform amplitudes (mean ± SE, six mice per group) from wild-type (+/+ ) and AQP4-null (−/−) mice in CD1 and C57/bl6 genetic backgrounds. Left: Absolute amplitudes. Right: Paired analysis of relative amplitudes shown as percentage of basal (preischemia) amplitudes. *P < 0.05; **P < 0.001.

after ischemia, although the difference between wild-type and
AQP4-null mice was not significant.

To investigate whether altered ECS properties in the cellular
layers of retina could be responsible for the neuroprotective
effects of AQP4 deletion, we adapted a photobleaching
method used previously to demonstrate ECS expansion in
neocortical cortex of AQP4-deficient mice. In the current study,
photobleaching was performed on fluorescently stained retinal
slices. After embedding retinal tissue in agarose, we cut 200-
µm-thick slices on a Vibratome for staining of the ECS with
4-kDa FITC-dextran. After the excess dye was rinsed away, the
slice was placed on a porous filter in contact with oxygenated
aCSF and covered with mineral oil. Figure 5A shows a retinal
slice viewed by transmitted light microscopy, in which the cellular
inner and outer nuclear layers could be identified for
photobleaching measurements. Under the conditions of the
experiment (35°C, oil covered, oxygenated aCSF) slices re-
mained viable for at least 10 to 15 minutes, as judged from vital
dye exclusion, absence of cell swelling, and constancy of ECS
diffusive properties.

FITC-dextran diffusion in the ECS of the INL and ONL was
measured from the time course of fluorescence recovery after
photobleaching of a circular spot. Recovery was measured in
the same spot using an attenuated laser beam that did not itself
cause photobleaching. Figure 5B shows representative fluores-
cence recovery curves in aCSF, and in INL and ONL. The
fluorescence recovery curves in retinal slices from wild-type
and AQP4 null mice were nearly superimposable. Figure 5C
summarizes half-times for fluorescence recovery (t1/2; See the
Methods section) with each point (open circles) summarizing
averaged data for slices from a different mouse. There was no
significant AQP4-dependent difference in FITC-dextran diffu-
sion in the ECS of the INL or ONL in retinal slices.

DISCUSSION

We found significant retinal protection after ischemia in mice
deficient in the Müller cell water channel AQP4. Retinal ischemia
results in irreversible morphologic and functional changes due to the deprivation of glucose and oxygen, followed by a cascade of biochemical responses involving glutamate release, water and ion transport, and activation of apoptosis-signaling pathways. As discussed in the introduction, the rationale for study of retinal neuroprotection in AQP4 deficiency was protection of brain tissue in AQP4 deficiency after ischemic stroke produced by middle cerebral artery occlusion. AQP4-expressing glial cells in brain have a similar supportive relationship to neurons as do the AQP4-expressing Müller cells in retina to ganglion cells and bipolar cells. The ECS in retina contains ions, neurotransmitters, and various matrix macromolecules, forming the microenvironment bathing Müller cells, bipolar cells, and ganglion cells, and facilitating cell-cell communication by diffusible solutes. On theoreti-

FIGURE 4. Retinal morphology after ischemia. (A) Representative hematoxylin and eosin-stained retinal sections before and at 12 and 96 hours after 60 minutes of retinal ischemia in wild-type and AQP4-null mice (CD1 background). Note the retinal swelling at 12 hours and degeneration at 96 hours. Scale bar, 100 µm. (B) Cell counts in the ONL, INL, and GCL before (top) and at 96 hours (bottom) after 60 minutes ischemia in CD1 mice. Counts (mean ± SE, three mice per group) expressed as number of stained nuclei per 100-µm length of retinal sections. (C) Average thickness of retinal layers: ORL, IRL, INL, and ONL (mean ± SE, 12 +/-/+ and 8 -/-/− mice at 96 hours after 60 minutes ischemia in CD1 mice. Counts (mean ± SE, three mice per group) expressed as number of stained nuclei per 100-µm length of retinal sections. (C) Average thickness of retinal layers: ORL, IRL, INL, and ONL (mean ± SE, 12 +/-/+ and 8 -/-/− mice at 96 hours after 60 minutes ischemia in CD1 mice. Counts (mean ± SE, three mice per group) expressed as number of stained nuclei per 100-µm length of retinal sections. (C) Average thickness of retinal layers: ORL, IRL, INL, and ONL (mean ± SE, 12 +/-/+ and 8 -/-/− mice at 96 hours after 60 minutes ischemia; 5 +/-/+ and 4 -/-/− mice at 96 hours after 45 minutes ischemia; 5 mice per group at other time points). *P < 0.05; **P < 0.005.
cell water permeability and \( K^+ \)/H11001 source is at the IPL and the sink in the OPL.48 The OPs are believed to arise from the IPL 49 and are dependent on the ical grounds, AQP4 deletion in Müller cells and reduced Müller cell water permeability is likely to be reduced by AQP4 deletion as well as altered ECS volume and composition after ischemia. As in other electrically excit-
ciable tissues, cell swelling and altered ECS homeostasis may be important early determinants of retinal neuronal cell injury and apoptotic cell death. We used a well-established model of retinal ischemia-reperfusion produced by transient elevation of intraocular pressure.50 AQP4 deficiency did not reduce the permeability of the retina after ischemic damage in AQP4 deficiency,35 we found no difference in fluorescence probe diffusion in the ECS of retinal cellular layers. Thus, altered basal ECS properties are unlikely to be involved in the mechanism of retinal protection after ischemia in AQP4-null mice. There are many possible explanations for altered ECS volume in AQP4 deficiency in cerebral cortex but not in retina, such as differences in cell transport mechanisms, and tissue architecture, and compliance.

The molecular-level mechanism by which AQP4 deficiency in mice protects the retina against ischemic damage thus remains unclear. As mentioned above, neither altered Müller cell swelling nor differences in baseline ECS properties were found in AQP4 deficiency. Although differences in ECS may occur after ischemia, it would be difficult to determine whether such differences are primary or secondary. A recent study by Dalloz et al.52 showed that targeted disruption of the dystrophin gene in mice results in greater ganglion cell death after transient ischemia, as well as altered cellular distribution of AQP4 and Kir4.1. The mechanisms responsible for their observation are probably very different from the opposite finding in the current study of retinal protection in AQP4 deficiency, where no impairment of Kir4.1 cellular expression or membrane targeting was found. Based on recent literature, other possible explanations for the retinal protection found in the present study include altered glutamate release and re-
other possible explanations for the retinal protection found in the present study include altered glutamate release and re-


diffusion of FITC-dextran in retinal slices. (A) Transmitted light image of a retinal slice. (B) FRAP of retinal slices after ECS staining with 4-kDa FITC-dextran. Representative recovery curves in retinal slices from wild-type (+/-) and AQP4-null (/-) mice, along with the aCSF control (50 mg/mL FITC-dextran in aCSF) and (C) Averaged half-times for fluorescence recovery (t_{1/2}) in ONL and INL for measurements in (B). Each data point (circles) represents the mean \pm SE for three or more recovery curves measured on slices from individual mice with averaged data (squares) for each condition. Differences were not significant.

A small baseline reduction in amplitudes of b-waves and OPs was found in ERG measurements in C57/bl6 but not CD1 mice. The b-wave is believed to arise from bipolar cell depo-
larization and K^+ channel activation in Müller cells.12,45 Current source-density analysis has suggested that the b-wave source is at the IPL and the sink in the OPL.46 The OPs are believed to arise from the IPL and are dependent on the retinal microcirculation.49 AQP4 deficiency did not reduce the amplitude of a-waves, which are produced primarily by the photoreceptors.

Transient retinal ischemia produced retinal swelling in the first 6 to 12 hours, primarily in the IPL. However, the extent of initial retinal swelling was not significantly different in wild-
type versus AQP4-null mice. Although Müller cell osmotic water permeability is likely to be reduced by AQP4 deletion as found for brain astroglia,51 the similar retinal swelling in wild-
type and AQP4-null mice is not surprising because cellular osmotic equilibration even in the absence of aquaporins occurs in <1 minute, a time scale much shorter than that for the ionic-solute routes responsible for cell swelling and volume regulatory phenomena. Retinal architecture was disrupted at later times with preferential thinning of the INL and IPL. The thinning and loss of cellularity in the internal retina was signif-
ificantly reduced in AQP4 deficiency, with corresponding pres-
ervation of retinal function, as quantified from b-wave and oscillatory potential amplitudes.

In conclusion, AQP4 deletion in mice conferred significant preservation of retinal function and architecture after retinal ischemia. The neuroprotective effect of AQP4 gene disruption suggests the possibility of AQP4 inhibition as a novel approach to limit retinal injury after ischemia produced by vascular occlusive and ischemic diseases.

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References


