Reduced Expression of Aquaporin-9 in Rat Optic Nerve Head and Retina following Elevated Intraocular Pressure

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PURPOSE. To investigate the effect of chronically elevated intraocular pressure (IOP) on the expression of water channel aquaporins (AQPs) 1, 4, and 9 in the optic nerve and retina in rats.

METHODS. Three episcleral veins were cauterized to elevate IOP in the left eyes of Sprague-Dawley rats. IOPs were monitored with a rebound tonometer. At 2 and 4 weeks after surgery, eyeballs with the attached optic nerve were enucleated for cryosectioning with immunohistochemistry, or dissected retinas and desheathed optic nerves were subjected to gene expression analyses.

RESULTS. IOP was significantly increased after surgery up to 4 weeks (P = 0.0008). In the control optic nerve, the unmeylinated portion showed only AQ9P immunoreactivity, whereas the myelinated portion expressed both AQ4P and AQ9P immunoreactivities colabeled for glial fibrillary acidic protein but not for neurofilament. In the control retina, AQ9P1 was expressed in the outer nuclear layer and photoreceptors, AQ4P was expressed in Müller cell endfeet, and AQ9P was expressed primarily in NeuN-positive cells in the ganglion cell layer (GCL). Elevated IOPs substantially reduced AQ9P9 expression in the optic nerve head (ONH) and the GCL and decreased the retinal gene expression, but not immunoreactivity, of AQ9P1.

CONCLUSIONS. AQ9P9 was the only water channel expressed in the unmeylinated portion of the ONH and in the GCL whose expression was reduced after IOP elevation. Given that AQ9P9 presumably acts as a channel for metabolites to pass from astrocytes to neurons, the reduced expression of AQ9P9 at these specific sites may be implicated in the pathogenesis of glaucomatous optic neuropathy. (Invest Ophthalmol Vis Sci. 2010; 51:4618–4626) DOI:10.1167/iovs.09-4712

Glaucoma is a progressive optic neuropathy characterized by optic nerve axonal degeneration, optic nerve head (ONH) excavation, and visual field defects. Elevated intraocular pressure (IOP) is one of the definitive risk factors for the development and progression of glaucomatous optic neuropathy. Evidence is accumulating that both glaucoma in humans and experimentally induced elevation of IOP in animals cause apoptotic death of retinal ganglion cells (RGCs) and glial activation in the retina and the ONH. However, the relationship between RGC apoptosis and glial reactivity induced by elevated IOP remains to be investigated.

Aquaporins (AQPs) are a family of integral membrane proteins that allow water to cross the plasma membrane. They are critically involved in the maintenance of ionic and osmotic balance in the central nervous system (CNS) in response to osmotic gradients and differences in hydrostatic pressure. Among the 13 isoforms of the mammalian AQP protein family identified thus far, at least four AQPs are expressed in the retina: AQ9P0, AQ9P1, AQ9P4, AQ9P9. AQ9Ps 0, 1, and 9 are reportedly expressed specifically in subpopulations of neurons whereas glial cells (Müller cells and astrocytes) show polarized expression of AQ9P4. In contrast, in the optic nerve, AQ9P4 is the only water channel that was reportedly heterogeneously expressed by fibrous astrocytes. Among the four AQPs identified in the retina, AQ9Ps 0, 1, and 4 are classified as classical AQPs that are selective water channels, whereas AQ9P9 is an aquaglyceroporin that is also permeable to a wide variety of noncharged solutes such as lactate and glycerol. Alterations of the amount and/or location of retinal expression of AQPs 1 and 4 have been intensively investigated in a variety of pathologic conditions such as ischemia/reperfusion and diabetes. Growing attention has been paid to the functional significance of AQ9P9 as a lactate-glycerol channel in the CNS because both lactate and glycerol can serve as fuel for neurons. Further, recent studies have provided evidence that in addition to their well-known roles as water channels, AQP proteins may play a critical role in the migration of astrocytes and the regulation of neuronal apoptosis. Despite the potential relevance to the pathogenesis of glaucomatous optic neuropathy, only a few studies have evaluated the role of AQPs in pathologic changes in glaucoma models.

The purpose of this study was to investigate the effect of elevated IOP on the expression of AQP genes and proteins in the retina and the optic nerve in a rat model of chronic ocular hypertension induced by episcleral vein cauterization (EVC), which has been proven by our group and others to accelerate RGC apoptosis and to enhance glial activation in the ONH and retina.

MATERIALS AND METHODS
All animal experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Kobe University Graduate School of Medicine.

Animal Handling and Induction of Elevated IOP
Male Sprague-Dawley rats (weight range, 250–300 g) from CLEA Japan (Osaka, Japan) were used. All animals were housed in the Kobe
University animal facility with ad libitum access to food and water under a 12-hour light/12-hour dark cycle at room temperature (24 ± 2°C). Induction of elevated IOP by EVC was performed on left eyes essentially according to a method developed by Shareef et al. and as described in our previous reports. In brief, rats were anesthetized by intraperitoneal injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (10 mg/kg). After minimum conjunctival incision, three episcleral veins near the superior and temporal rectus muscles were cauterized by diathermy. The eyes were flushed with saline and treated with antibiotic ointment.

IOPs were measured in both eyes of awake rats using a rebound tonometer (TonoLab; Tiotol, Helsinki, Finland) after topical application of 0.1% proparacaine; this was performed essentially according to Wang et al. and Morrison et al. In brief, the instrument was clamped to a ring stand with the probe oriented horizontally. Rats placed on an adjustable table were positioned, and the height of the table was adjusted to locate the probe tip at the center of the cornea at a 2-mm distance. In each recording session, the tonometer took six measurements deemed reliable by internal software that eliminated the highest and lowest readings and then generated and displayed a mean. After recording five consecutive sessions, we averaged the five mean IOPs; this was defined as the IOP at the specific time point.

At 2 weeks and 4 weeks after EVC, anesthetized rats were transcardially perfused with 4% paraformaldehyde. Eyeballs with attached optic nerves were removed, embedded in 20% sucrose optimal cutting temperature compound, snap-frozen, and stored at −80°C until use for cryosection. In some groups of rats, retinas lacking the ONH and optic nerves, including the ONH (from which the myelin sheath was removed carefully), were separately dissected and immediately subjected to real-time reverse transcription (RT) quantitative (q) polymerase chain reaction (PCR). Left eyes of sham-operated age-matched rats without EVC were used as controls. We did not use contralateral eyes as controls because we noticed previously that in rodents, glial activation occurred not only in ipsilateral but also in contralateral retinas in response to injury at the long-term follow up; thus, the contralateral eyes did not seem to be real controls.

Immunohistochemistry

Sagittal cryosections (8 μm) were collected on silane-coated slides, yielding approximately 10 slides with attached ONH per eye and additional slides with sections of the adjacent retina. After blocking with 10% goat serum in PBS and 0.3% Triton (blocking solution), the slides were incubated with primary antibodies in blocking solution overnight at 4°C. The primary antibodies used were mouse anti-glia fibrillary acidic protein (GFAP, 1:400; Millipore, Billerica, MA), mouse anti-neurofilament 200 (NF, 1:400; Sigma-Aldrich Japan, Osaka, Japan), mouse anti-glutamine synthetase (GS, 1:400; Millipore), mouse anti–protein kinase C α (PKCa, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-neuronal nuclei (NeuN, 1:100; Millipore), rabbit anti-AQP1 (1:200; Millipore), rabbit anti-AQP4 (1:200; Santa Cruz Biotechnology), and rabbit anti-AQP9 (1:200; Santa Cruz Biotechnology or Alpha Diagnostics International, Inc., San Antonio, TX). After extensive washing in PBS with 0.3% Triton, the slides were incubated with secondary antibodies together with iodide (ToPro-3; Invitrogen Corp., Carlsbad, CA) for 1 hour at room temperature. The secondary antibody was fluorescein isothiocyanate-conjugated Fab’2 fragment anti-mouse IgG or tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit IgG (AfiniPure; Jackson ImmunoResearch, West Grove, PA). After extensive washes, the slides were coverslipped with aqueous mounting medium (PermaFluor; Laboratory Vision Corporation, Fremont, CA). Images were captured on a personal computer linked to a confocal laser scanning microscope system (LSM 5 Pascal; CarlZeiss, Thornwood, NY).

Real-time RT-qPCR

Total RNA was extracted from retinas (n = 5 each for control eyes, eyes with elevated IOP, and contralateral eyes) and desalted optic nerves with ONH (control eyes, n = 4; eyes with elevated IOP, n = 4; contralateral eyes, n = 5) using a purification kit (RNeasy Mini Kit; Qiagen Valencia, CA) according to the manufacturer’s instructions. The amount of total RNA was quantified with a spectrophotometer (SmartSpec; Bio-Rad Laboratories, Inc., Hercules, CA). Total RNA was reverse transcribed using a reverse transcription kit (QuantiTect; Qiagen) according to the manufacturer’s instructions. Quantitative PCR was performed using gene expression assays (TaQMan; Applied Biosystems, Foster City, CA) on a real-time PCR system (StepOnePlus; Applied Biosystems).

Sense and antisense primers for the real-time RT-qPCR experiments were specifically designed by scientists at Applied Biosystems from the coding regions of each AQP gene. Because of proprietary issues and the policy of Applied Biosystems, the exact primer sequences used for the real-time RT-PCR experiments are not provided but can be requested from the company based on the following information: AQP1 (assay ID, Rn00562834_m1; GenBank accession number, NM_012727.1; target exon, 1), AQP4 (assay ID, Rn00563196_m1; GenBank accession number, NM_012825.2; target exon, 2), AQP9 (assay ID, Rn00576311_m1; GenBank accession number, NM_022960.2; target exon, 5). Glyceraldehyde phosphate dehydrogenase (GAPDH; assay ID, Rn99999916_s1; GenBank accession number, NM_017008.3; target exon, 3) was used as an internal control. Thermal cycling conditions consisted of 40 cycles of denaturation at 95°C for 1 second and annealing and extension at 60°C for 20 seconds.

Samples were run in triplicate. Fold change (FC) calculation was performed with the comparative threshold cycle (Ct) or the ΔΔCt method, based on the formula FC = 2−ΔΔCt, to calculate normalized gene expression FCs in test samples relative to a calibrator sample (i.e., EVC samples compared with control samples). The first step in FC analysis was normalization of target gene expression signal against GAPDH gene expression (ΔCt). The second step was to calculate the difference between normalized target gene expression in EVC and control samples (ΔΔCt). FC calculation, which represents the difference in gene expression level between EVC samples and control samples, was carried out for each gene individually using the formula described.

Statistical Analysis

Numerical data were expressed as mean ± SD. Time course of changes in IOP between EVC eyes and contralateral eyes were compared with paired t-test, whereas those between EVC eyes and control eyes were compared with unpaired t-test. For quantifying the ratio of AQP9 immunoreactive cells among NeuN-positive cells in the ganglion cell layer (GCL), 10 randomly selected retinal sections per eye were captured with the highest magnification (200×). Means of four eyes each of controls and 4 weeks after EVC were compared using the unpaired t-test. Messenger RNA expression levels of retina and ONH were compared between elevated IOP eyes and control eyes and between contralateral eyes and elevated IOP eyes using unpaired t-test.

Statistical analyses were performed with statistical software (StatView, version 5.0; SAS Institute, Cary, NC). All statistical values were judged significant if P < 0.05.

Results

Time Course of Changes in IOP

Cauterization of three episcleral veins resulted in a sustained increase in IOPs (Fig. 1), as previously reported. The average IOP of awake rats before EVC was approximately 17.6 ± 0.9 mm Hg. EVC increased IOPs to 36.4 ± 4.8 mm Hg at 1 week after EVC, when the IOPs of contralateral and control eyes were 16.3 ± 1.2 mm Hg and 17.5 ± 0.8 mm Hg, respectively. The IOPs of EVC eyes gradually decreased over time but were significantly higher than those of contralateral and control eyes during the entire experimental period up to 4 weeks (Fig. 1).
Immunohistochemical Findings in ONH and the Effect of Elevated IOP

The rodent ONH can be divided into three regions: the neck region, located at the level of the sclera; the transition region, an extension zone between the neck region and the myelinated retrobulbar optic nerve; and the retrobulbar region, characterized by myelinated axons.38–40

Immunolabeling for AQPs 1, 4, and 9 in ONH cryosections of control rats revealed a characteristic immunoreactivity pattern for AQPs (Figs. 2–5). AQP1 immunoreactivity was detected in the outer retinal region, which is shown in more detail later, and in the extraocular connective tissue but was not exhibited in the optic nerve from the neck region through the transition to the retrobulbar region (Figs. 2, 3). Although the neck and the transition regions, each of which consisted of unmyelinated axons, lacked AQP4 immunoreactivity, the reti-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/)  
**Figure 1.** Time course changes in IOP after episcleral vein cauterization. Data represent mean ± SD. *P < 0.0001 between eyes with EVC and contralateral eyes (paired t test) and between eyes with EVC and control eyes (unpaired t test). **P = 0.0008 between eyes with EVC and contralateral eyes.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/)  
**Figure 2.** Immunolabeling for three AQPs in the control ONH cryosections. Negative control had no primary antibodies and was counterstained. ILM, inner limiting membrane; S, sclera; C, choroid; N, neck region; T, transition region; R, retrobulbar region. Asterisk: extraocular vein. Scale bar, 50 μm.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/)  
**Figure 3.** Immunolabeling for AQP1 and GFAP in ONH cryosections. Arrowheads: vitreal surface of retina. Arrows: neck and transition region of ONH. Scale bar, 50 μm.
robulbar myelinated optic nerve exhibited intense AQP4 immunoreactivity (Figs. 2, 4). These are consistent with previous observations by Nagelhus et al.¹⁰ and Li et al.²⁰ In contrast, an intense honeycomb pattern of AQP9 immunoreactivity was detected throughout the optic nerve from the vitreo-ONH interface to the retrobulbar myelinated region (Figs. 2, 5). Thus, the unmyelinated ONH portion expressed only AQP9 among the AQP proteins tested.

In control eyes, sharp and linear GFAP immunoreactivity at the vitreo-ONH interface was contiguous with the honeycomb pattern of immunoreactivity within the optic nerve (Figs. 2–5).¹⁴⁻¹⁰⁻⁴⁰ whereas streaming NF immunoreactivity along with the axonal projections was observed continuously from the nerve fiber layer of retinas to the optic nerve (Fig. 6).⁴¹ Coimmunolabeling with GFAP or NF disclosed that AQP9 expression in all optic nerve regions and AQP4 expression in the retrobulbar optic nerve region was colocalized only with GFAP (Figs. 2–5) but not with NF (Fig. 6), indicating that AQPs 4 and 9 were expressed by astrocytes, but not by nerve axons, in the optic nerve.

Elevated IOPs led to no obvious change in immunoreactivity patterns for AQP1 and 4 and GFAP in the optic nerve at either 2 or 4 weeks after EVC (Figs. 3, 4). In comparison, AQP9 immunoreactivity in the optic nerve was reduced after IOP elevation (Figs. 5, 6). The reduction was more prominent 4 weeks rather than 2 weeks after IOP elevation (Fig. 5), as evident in the merged pictures of coimmunolabeling for AQP9 and GFAP in which the yellow merged color in controls (Figs. 2, 5, upper panel) became weaker at 2 weeks (Fig. 5, middle panel) and was eventually replaced with the green monocolor at 4 weeks after EVC (Fig. 5, lower panel). Elevated IOPs also reduced NF immunoreactivity, indicating the loss of nerve fibers (Fig. 6). No obvious differences were detected in ONH AQP 1, 4, and 9 immunoreactivity between control eyes and eyes contralateral to EVC (data not shown).

Immunohistochemical Findings in Retina and the Effect of Elevated IOP

Coimmunolabeling for AQP1 and GFAP together with nuclear staining (ToPro-3; Invitrogen Corp.) disclosed that AQP1 was expressed exclusively in the outer nuclear layer (ONL) and the photoreceptor layer (PRL), irrespective of IOP status (Fig. 7).

AQP4 was predominantly expressed at the outer plexiform layer (OPL) and the vitreoretinal border and around the vessels residing in the inner retina and partially in the processes vertically spanning the retina (see Fig. 8). Coimmunolabeling with two glial markers (Gs and GFAP) disclosed AQP4 expression by astrocytes surrounding the superficial vessels and by the Müller cell processes spanning the retinal parenchyma (see Fig. 8). These observations corroborate previous findings.⁹⁻¹³,¹⁶⁻¹⁹ Elevated IOP consistently up-regulated GFAP immunoreactivity spanning the retina (Figs. 7, 8) as seen in retinas under a variety of stresses,⁴⁻⁶ although those did not alter the AQP4 immunoreactivity pattern (Fig. 8).

In control retinas, there was a linear pattern of AQP9 immunoreactivity spanning the retina (Figs. 9, 10) and round
AQP9 immunoreactivity at the GCL (Fig. 10). The former was partially coimmunolabeled with the Müller cell marker GS, both at the inner plexiform layer (IPL) and the ONL (Fig. 9, upper panel) and with the marker for rod bipolar cells and a heterogeneous population of amacrine cells, PKCa, at the INL (Fig. 9, lower panel), indicating AQP9 expression in these cells. AQP9 immunoreactivity at the superficial retinal layer was sparse in contrast to the AQP4 immunoreactivity. This held true even for coimmunolabeling with GFAP instead of GS (data not shown). Thus, retinal astrocytes unlikely express AQP9. On the other hand, the round AQP9 immunoreactivity at the GCL was colocalized with NeuN immunoreactivity (Fig. 9), indicating the neuronal expression of AQP9, which is compatible with the observation by Dibas et al.15 Here we show the results using an AQP9 antibody provided by Santa Cruz Biotechnology only. However, a similar pattern of retinal AQP9 immunoreactivity was also detected using the antibody provided by Alpha Diagnostics (data not shown).

Although elevated IOPs did not affect the linear pattern of AQP9 immunoreactivity (Fig. 10A), neuronal AQP9 expression at the GCL was substantially reduced (Figs. 10B, 10C). When the relative ratio of AQP9 immunoreactive cells among NeuN immunoreactive cells were counted, 91.9 ± 1.8% NeuN-positive cells were AQP9 immunoreactive in the control retinas, which was reduced to 22.9 ± 7.1% in the retinas 4 weeks after IOP elevation (P < 0.001; Figs. 10B, 10C).

No obvious differences in retinal AQP1, 4, and 9 expression between control eyes and eyes contralateral to EVC were observed (data not shown).

**Effect of Elevated IOP on mRNA Expression of AQP1, 4, and 9 in the ONH and Retina**

We measured alterations in the mRNA expression of AQP1, 4, and 9 compared with those of GAPDH in the ONH and retina after IOP elevation induced by EVC (Fig. 11). In the ONH, there was no significant change in AQP1 and 4 gene expression between controls and eyes with elevated IOP, whereas AQP9 mRNA levels in eyes with elevated IOP at 4 weeks after EVC were reduced to approximately 50% of the level in controls (P < 0.05).
On the other hand, in the retina, mRNA levels of AQPs 4 and 9 were not statistically different between controls and eyes with elevated IOP, whereas AQP1 gene expression in eyes with elevated IOP at 4 weeks after EVC was reduced to approximately two-thirds the level in controls ($P < 0.05$). There was no significant difference in mRNA levels of AQPs 1, 4, and 9 between controls and eyes contralateral to EVC (data not shown).

**Discussion**

The present study has several important findings regarding the expression of AQP water channels both in controls and in eyes with elevated IOP. First, astrocytes in the optic nerve of control rats showed a unique distribution of the expression of AQPs in that AQPs 4 and 9 were the predominantly expressed AQP proteins. In addition, AQP9 was the only water channel expressed by...
astrocytes in the unmyelinated part of the ONH (i.e., in the neck and transition regions). Second, we confirmed previous observations regarding basal retinal expression of the AQPs. AQP1 is expressed predominantly in the outer retina,\textsuperscript{8,9,12,13,16} AQP4 in the perivascular and vitreal endfeet of Müller cells and in astrocytes in the inner retina,\textsuperscript{10,10.14–15} and AQP 9 in the somata of neural cells in the GCL.\textsuperscript{15} Third, elevated IOP reduces gene and protein expression of AQP9 in ONH astrocytes and neuronal AQP9 immunoreactivity in the retina, whereas expression of AQPs 1 and 4 both in the ONH and in the retina are not altered by IOP elevation.

Water homeostasis is critical for maintaining physiological neuronal activity in the CNS, including the retina and optic nerve, because water movement is coupled to ionic currents that are the basis for neuronal excitability.\textsuperscript{8,9,21} In this regard, the aquaporin family is thought to participate in the regulation of neuronal activity primarily by supporting efficient ion clearance.\textsuperscript{8,9,21} In addition, it has been suggested that AQP9 may play a pivotal role in energy metabolism of the brain.\textsuperscript{14,15,21} As mentioned earlier, AQP9 has a broad substrate specificity and can transport noncharged osmolytes such as glycerol, lactate, and \( \beta \)-hydroxybutyrate.\textsuperscript{8,9,14,15,21} In the rodent brain, astrocytes in the white matter tract\textsuperscript{21,44} have been found to express AQP9. Because glycerol and lactate can also be used as an energy substrate by neurons,\textsuperscript{22,45} brain AQP9 may act as a metabolite channel for lactate and glycerol delivery to and from astrocytes.\textsuperscript{21} In a lactate shuttle model, glucose, the classical energy source for neurons, is transformed by astrocytes into lactate and diffuses from astrocytes to neurons,\textsuperscript{21} constituting an alternative energy substrate for neurons,\textsuperscript{22,45} brain AQP9 may contribute to lactate clearance from the extracellular space under ischemic conditions, resulting in lactic acidosis.\textsuperscript{43} Such an intimate distribution of AQPs 4 and 9 may reflect the synergistic action of these proteins in water and ionic homeostasis in the rodent brain.\textsuperscript{46} From this point of view, the presence of only AQP9 in the ONH suggests the vulnerability of this specific region in the optic nerve, which is thought to be the primary site for injury in glaucoma,\textsuperscript{4} to ischemic, osmotic, or metabolic imbalance, whether primary or secondary.

Gene and protein expression of AQP9 was substantially reduced in astrocytes in the optic nerve after IOP elevation rather than in a duration-dependent fashion. Although the functional significance of the reduced expression of AQP9 in the ONH after elevated IOP is not known, this reduction may be implicated in axonal degeneration through the inefficient transfer of energy substrates from fibrous astrocytes to axons. A previous study\textsuperscript{47} demonstrated that AQP9 expression was transiently decreased in astrocyte cultures under hypoxia. On the other hand, focal transient ischemia in the rodent brain induced by arterial occlusion was shown to upregulate AQP9 expression in astrocytes in the peri-infarct and swollen areas.\textsuperscript{35} AQP9 may contribute to lactate clearance from the extracellular space under ischemic conditions, resulting in lactic acidosis.\textsuperscript{43} The discrepancy of alterations in AQP9 expression between glaucomatous eyes in the present study and ischemia in the brain white matter may be attributed to the different types of injuries and suggest that the pathophysiology of axonal damage caused by elevated IOP is distinct from a pure ischemic stress.

In contrast to the homogeneous AQP9 immunoreactivity in optic nerve astrocytes of control rats, retinal astrocytes do not seem to express AQP9 protein. Instead, Müller cells exhibit AQP9 at their processes. It is generally accepted that there are two types of astrocytes: protoplasmic and fibrous.\textsuperscript{4,10,40} The former are located in the gray matter and have endfeet that contact synapses and blood vessels, whereas the latter are located in the white matter and have endfeet that contact nodes of Ranvier and blood vessels.\textsuperscript{4,40} In the brain, these two astrocyte types may originate from different progenitors.\textsuperscript{4,40} Based on this classification, retinal astrocytes that are located

![](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/ on 04/01/2017)
adjacent to RGCs and superficial vessels belong to the protoplasmic category, whereas optic nerve astrocytes that surround nerve axons belong to the fibrous category. This difference in origin may result in distinct protein expression and distinct responses to stress.

There are only two other reports of neuronal AQP9 expression in the rodent retina. Landiev et al. found that tyrosine hydroxylase-positive amacrine cells in stratum 1 of the IPL were exclusively immunoreactive for AQP9, which may be consistent with an enrichment of AQP9 in catecholaminergic neurons of the substantia nigra. Conversely, Dibas et al. showed immunocolocalization of AQP9 with the marker Thy-1 in the GCL of rat retinas and in RGC-5 cells, an immortalized cell line that originated from rodent RGCs. The present data may corroborate both findings, because the NeuN-positive cells at the GCL and some of the PKCα-expressing cells are immunoreactive for AQP9. The mode of expression changes of AQP9 in retinal neurons may vary in an injury type-dependent manner, as observed in astrocytes, because ischemia/reperfusion did not alter AQP9 expression in the amacrine cells,

whereas hypoxia and hypotonic shock upregulated AQP9 gene expression in RGC-5 cells. In contrast, the present study demonstrated that elevated IOP reduced the number of NeuN-immunoreactive cells that also expressed AQP9 in the GCL. It has been generally accepted that RGCs undergo apoptotic cell death both in humans with glaucoma and in animals with experimentally induced elevated IOP. Increased evidence suggests that AQPs may play a role in the apoptotic death of neurons by decreasing the cellular volume; this phenomenon is termed apoptotic volume decrease. Such activity represents the cellular shrinkage that is the initial step of apoptosis processes and requires the movement of water out of cells. It will be a task for future experiments to determine whether decreased expression of AQP9 in RGCs triggers RGC apoptosis through inefficient transfer of energy substrates from astrocytes, as mentioned, or if it reflects a compensatory response to the apoptotic stimulus that reduces the chance of apoptotic volume decrease.

Few studies have investigated the effect of elevated IOP on AQP4 expression in the ONH. Johnson et al. analyzed the gene expression of AQP4 by RT-qPCR in the ONH, with elevated IOP induced by the injection of hypertonic saline into episcleral veins. They found an approximately 50% reduction of AQP4 mRNA levels in eyes with elevated IOP compared with contralateral eyes. However, they also stated that the degree of reduction was not linearly correlated with the magnitude of optic nerve injury scores. Using the same glaucoma model, Dibas et al. examined changes of AQP4 immunoreactivity in the ONH and described that elevated IOP enhanced AQP4 expression. In this condition, AQP4 immunoreactivity was present in the neck region of the ONH, not only in eyes with elevated IOP but also in the control fellow eyes. This is contradictory to the findings of previous studies by Nagelhus et al. and Li et al. and those of the present study. The discrepancy in the mode of alteration of AQP4 expression in the ONH after IOP elevation found among studies may be derived from the difference in the animal models used (episcleral vein hypertonic saline injection vs. episcleral vein catarization), the difference in the rat strains used (pigmented Brown Norway rats vs. albino Sprague-Dawley rats), and the duration of IOP elevation.

Regarding the retinal expression of AQPs 1 and 4, a variety of disease model result in distinct patterns of expression. For example, in ischemia/reperfusion injury and diabetes, AQP1 and AQP4 expression in astrocytic endfeet at the vitreoretinal border and around superficial vessels switched from AQP4 to AQP1. AQP1 gene expression was upregulated in isolated Müller cells taken from the retinas of rats with chemically induced diabetes. Form-deprivation myopia and combustion smoke exposure increased perivascular AQP4 expression and AQP4 gene expression, respectively. In this study, there was no apparent alteration in retinal immunoreactivity of AQPs 1 and 4 despite the reduced AQP1 gene expression after IOP elevation. Similar dissociation between reduced gene expression and sustained protein expression was observed for AQP4 in a rat model of branch retinal vein occlusion. These results suggest that retinal glial expression of water-selective AQPs may be affected by type and duration of injury. Further studies are needed to elucidate the functional significance of alterations in glial AQP expression in the retina in a variety of injuries.

In conclusion, the aquaglyceroporin AQP9 was the sole water channel expressed in astrocytes in the unmyelinated portion in the ONH and in neurons in the GCL whose expression was reduced after IOP elevation. Given that areas with AQP9 expression are the primary sites for glaucomatous injury and that AQP9 presumably acts as a metabolite channel from astrocytes to RGCs, the reduced expression of AQP9 may be implicated in the pathogenesis of glaucomatous optic neuropathy.

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