Changes in retinal aquaporin-9 (AQP9) expression in glaucoma

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Synopsis

The eye contains numerous water channel proteins and the roles of AQPs (aquaporins) in the retina are blurred, especially under disease conditions. The purpose of this study was to investigate the expression of AQP9 gene and proteins affected by elevated IOP (intraocular pressure) in a rat model of glaucoma induced by intravitreous injection of hypertonic saline into the episcleral veins. The gene and protein expressions of AQP9 were investigated by real-time PCR and Western blotting. The immunoreactive expression of AQP9, AQP4 and GFAP (glial fibrillary acidic protein) in the optic nerve of rats exposed to experimentally elevated IOP was detected by immunofluorescence microscopy. The mRNA and protein expression levels of AQP9 were up-regulated in the retina of an animal model of glaucoma. The immunoreactivities of the AQP9, AQP4 and GFAP were also detected and increased in the optic nerve region. The expression of AQP9 was up-regulated in this glaucoma model and the immunoreactivities of the AQP4 and GFAP were also detected as co-localizing with AQP9 in the optic nerve region, indicating retina ganglion cells were surrounded by activated astrocytes. This may indicate that the injured neurons may rely on the astrocytes. The alterations of AQP expression may compensate the glaucomatous damage.

Key words: aquaporin, glaucoma, intraocular pressure, retina

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INTRODUCTION

Glaucoma is a group of slow progressive vision disorders affecting the trabecular meshwork, the ONH (optic nerve head) and RGCs (retinal ganglion cells) [1]. Glaucoma is classified as primary if evidenced by optic neuropathy and as secondary if evidenced by elevated IOP (intraocular pressure) caused by other pathological processes [2]. Most glaucoma patients have POAG (primary open angle glaucoma), which is related to elevated IOP [3,4]. Other risk factors for glaucoma are increasing age [5], being African–American [6], visual field abnormalities in visual field examinations [7], high myopia, family history of glaucoma [8], thin cornea and a cup-to-disk ratio greater than 0.4 [7].

Glaucoma patients have slow, progressive degeneration of RGCs. Factors contributing to RGC death may include local ischaemia–hypoxia [9], excess glutamate [10], low functional cellular pumps and glutamate transporters [11], oxidative stress and free radical formation [12], inflammatory cytokines such as TNF (tumour necrosis factor) [13], abnormal immunity and secondary neurodegeneration caused by neuronal environmental changes [14]. Even though glaucoma disease factors are not fully characterized, elevated IOP is the main factor without question. The pressure in the eye is the balance between AH (aqueous humor) secretion from the ciliary body and drainage via the trabecular meshwork and the canal of Schlemm. The mechanisms responsible for the pathophysiology of glaucoma are poorly understood, but early detection is very crucial because elevated IOP may cause optic nerve changes (cupping) that eventually lead to RGC death and glaucoma [15].

AQPs (aquaporins) are hydrophobic membrane proteins and the narrowest diameter of the pore of AQPs is 2.8˚Å (1˚Å = 0.1 nm) [16]. In addition, the transmembrane domains are mostly hydrophobic with NPA (asparagine–proline–alanine) regions. These two polar asparagines (N76 and N192) form...
hydrogen bonding with water molecules, whereas other residues repel protons. Thus, AQPs use space, electrostatic interactions and hydrogen bonding to control water movement through the pore. This explains the highly effective water transport function. The purpose of this study was to investigate the effect of elevated IOP on the expression of AQP9 genes and proteins in the retina and the optic nerve in a rat model of chronic hypertension induced by the injection of hypertonic saline into episcleral veins.

MATERIALS AND METHODS

The Morrison glaucoma model

The procedure for the rat glaucoma model was described previously by Morrison et al. [17]. Male Brown Norway rats weighing 200–300 g were used and fed standard nutrients. Rats were housed individually in cages under a controlled environment and maintained on a 12 h:12 h light–dark cycle and allowed tap water ad libitum. IOP was elevated in one eye by injecting 50 μl of 1.8 M saline into the episcleral veins of anaesthetized rats such that blanching was observed. After surgery, rats were housed under constant low light (<90 lux) to minimize effects of circadian influences on IOP. After application of a topical anaesthesia (0.1 % proparacaine) the rats were stabilized and conscious during IOP measurements which were taken with a Tonolab rebound tonometer (Colonial Medical Supply Co. Inc.). Rat eyes were dissected and either prepared for immunohistochemistry or processed for Western blotting and QPCR (quantitative PCR). The contralateral eye served as the control. All studies were conducted in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and guidelines of the University of Texas Health Science Center Committee on Animal Welfare.

Double immunofluorescence labelling studies

AQP9 and AQP4 antibodies were from Santa Cruz Inc and monoclonal anti-GFAP (glial fibrillary acidic protein) antibodies were from Neomarkers. Monoclonal anti-tubulin antibodies were from Upstate Inc and secondary antibodies (donkey anti-mouse conjugated Alexa Fluor® 633, goat anti-rabbit conjugated Alexa Fluor® 633 and goat anti-mouse conjugated Alexa Fluor® 488) were from Invitrogen Inc. Rat eyes were dissected, fixed in formalin, embedded in paraffin and 5 μm sections were obtained. Retina sections were de-paraffinized in xylene, rehydrated using ethanol and rinsed with PBS (Sigma-Aldrich Co.). Permeabilization was done with 0.1 % (v/v) Triton X-100 and non-specific binding was blocked by 5 % (w/v) BSA. The sections were incubated for 2 h at room temperature (25 °C) with a proper mixture of primary antibodies (1:100): AQP9, AQP4 and GFAP. After rinsing with PBS, sections were allowed to incubate for 1 h in the dark at room temperature in a mixture of appropriate secondary antibodies. Coverslips were mounted on glass slides in the anti-fade medium (FluorSave; Calbiochem) and allowed to dry in the dark. Images of sections were taken with a Zeiss LSM 410 confocal microscope.

Western blotting

Western blotting was performed on total retinal lysates. Total retinal lysate was prepared by dissecting retinas from the eyes and solubilizing them in 250 μl of a solution containing 20 mM Tris (pH 7.4), 10 % (w/v) sucrose, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 % (v/v) Triton X-100, 0.1 % (w/v) SDS and protease inhibitors at 4 °C. Retinal lysates were incubated for 30 min on ice and briefly sonicated before centrifugation at 14 000 g for 15 min. The supernatant was collected and protein concentration was measured by a BCA (bicinchoninic acid) protein assay kit (Sigma) using BSA as the standard.

Proteins were separated with SDS/10%PAGE, with 100 μg of protein loaded in each lane. Gels were equilibrated for 10 min in a transfer buffer and then electroblotted on nitrocellulose membranes for 75 min at 100 V. Western blotting was performed using the Amersham Chemiluminescent Kit. Membranes were incubated with 1 μg/ml of primary antibodies for 60 min and with a 1:10 000 dilution of secondary antibody for 30 min. Next, the membranes were exposed to an X-ray film before later development. Afterwards, the membranes were stripped and probed with anti-α-tubulin antibodies for normalization. Band densities were quantified with image-analysis software (Scion).

Real-time analyses

Retinas were dissected from the eyes, and total RNA was extracted with TRIzold® (Life Technology) by following the manufacturer’s instructions. In this study, 5 μg of total RNA was reverse transcribed using the iScript kit (Bio-Rad). QPCR was performed using primers listed below. Negative control QPCR reactions were performed in the absence of cDNA templates. β-Actin was used as a housekeeping gene. The primers for rat retinal AQP9 were 5′-CTCAGTCCCAGCTTCTCAC-3′ (sense) and 5′-CTCAGTCCCAGGCTTCTCAC-3′ (antisense), giving a 184-bp amplicon. The primers for retinal β-actin were 5′-TTGTGATGGTAGATGGTGTCAG-3′ (sense) and 5′-TTGTGATGGTACGAGTTCC-3′ (antisense), giving a 514-bp amplicon. The relative mRNA levels were determined by the comparative C_T (threshold cycle value) method as described in PE Biosystems User Bulletin #2. The fold change was determined by the following formula: Change = 2 – Δ(ΔC_T), where ΔC_T = C_T(target) – C_T(β-actin), and ‘target’ is the gene of interest. The first delta is the difference between the ΔC_T values of the treated eye and the control eye (ΔΔC_T) and represents the corrected shift of the analysed gene.

The running conditions of denaturation, annealing and extension were 95 °C (30 s), 60 °C (30 s) and 72 °C (60 s) for rAQP9 and 95 °C (60 s), 60 °C (60 s) and 72 °C (120 s) for β-actin.

To verify sequence of products, we performed regular PCR and ran the PCR products on a 1.5 % (w/v) agarose gel in parallel with 100 bp DNA markers before staining with ethidium bromide. The authenticity of PCR products was confirmed using a BLAST search of the sequence through the National Center for Biotechnology Information.
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Hypertonic saline was given in one eye of each Brown Norway rat to elevate IOP and the contralateral eye was allowed to serve as a control. Each data point represents the average of nine consecutive IOP measurements (means ± S.E.M.) for each eye in mmHg IOP values. In this study, nine Brown Norway rats were used for the respective recordings. *P < 0.001 between treated eyes and contralateral eyes (paired t test).

Statistical analyses

All experiments were repeated at least three times with up to four replicates per condition each time. Statistical significance was determined by using one-way ANOVA at \( P < 0.05 \).

RESULTS AND DISCUSSION

AQPs are involved in the homoeostasis of ions, osmolarity and volume inside a cell and in the ECM (extracellular matrix). These functions are fundamental in both the brain and spinal cord. Any imbalance can dramatically change neuronal signalling and even damage the brain. Dysfunctional water channel proteins can cause excess water accumulation, which affects the mortality of cells during a stroke, or brain damage caused by tumour, trauma or infection. For example, AQP4 is well known to be expressed at the BBB (blood–brain barrier) [18]. Brain oedema can be a result of up-regulation of AQP4 in astrocytes with or without disturbance of the BBB because AQP4 has an exceptionally high water permeability [19,20]. Astrocytes also express AQP9, which was up-regulated after transient brain ischaemia [21]. Because AQP4 expression is polarized in brain astrocytes when in contact with endothelial cells and neurons [22,23], it was no surprise that AQP4-null mice had better retinal structure and larger cell numbers than the wild-type mice after ischaemia [24].

Badaut et al. [25] were the first group to report water channels in neurons. Because catecholaminergic neurons respond to energy balance rather than osmotic pressure [26,27] and are located in the brain with other fuel-sensing neurons [28], AQP9 was expected to function in an energy-related role instead of a water-related role. In addition, both lactate and glycerol can be transported by AQP9, and both substances can be used as fuel by neurons [29,30]. Thus, the functional role of AQP9 in catecholaminergic and RGCs is still in debate.

The finding of AQP9 in the retina has been reported in recent years. Animal models of glaucoma have shown diversity of AQP expression [31–33]. The purpose of this study was to investigate the effect of elevated IOP, using the injection of hypertonic saline, on the expression of AQP4 and AQP9 genes in the retina in a rat model of chronic hypertension.

Injecting 50 μl of 1.8 M saline into the episcleral veins of anaesthetized rats resulted in elevated IOPs, as shown in Figure 1. The IOPs from untreated eyes of conscious rats, were in the range between 19 and 21 mmHg. The hypertonic saline injection increased IOP gradually from the first week. At the third week, the difference of IOP between the treated and untreated eyes on average was 10.6 mmHg and similar results were maintained for the fourth week. The IOP for the treated eyes had significant differences compared with those of untreated eyes during the entire experimental period (Figure 1).

Figure 2 shows both the gene and protein expression of AQP9 in the retina after the treatment compared with \( \beta \)-actin and \( \alpha \)-tubulin, respectively. Among these samples, the degrees of
AQP9 protein expression changes were different. It was clearly shown that the retina responded to the high IOP. Compared with Western blotting of the α-tubulin standard, the concentration of AQP9 was up-regulated in IOP, which had a level 2-fold more than detected in normal individuals. This comparison was made using the quantitative analysis software, and the P value was less than 0.05. As expected, the AQP9 mRNA level was higher in the group of treated eyes.

In the previous study, it was indicated that AQP9 is expressed in catecholaminergic amacrine cells in the rat retina. AQP9 was not found to be altered after ischaemia induced by transiently increased intracocular pressure [34]. However, AQP9 immunolabellings of the neuroretina and RPE (retinal pigment epithelium) were up-regulated by pressure-induced transient retinal ischaemia [33,36]. In another report, the level of AQP9 mRNA expression was increased in retina (not statistically different between controls and elevated IOP eyes), but decreased in the ONH due to the elevated IOP [31]. This may be as a result of chemical hypoxia, VEGF (vascular endothelial growth factor), high glucose, metabolic and oxidative stress. These changes may be involved in the adaptation of retinal cells to the development and resolution of retinal oedema [33,36].

GFAP is an intermediate filament and can be used as a cellular marker for retinal injury [37]. Previous studies indicated that increased expression of GFAP is thought to contribute to the mechanical strength of astrocyte, as well as enhanced cell survival [38]. Figure 3 shows the increase in expression of both AQP9 and GFAP in the region of the optic nerve. Several reports showed up-regulation of AQP9 in the experimental glaucoma models [39,40], and increased GFAP staining was observed in astrocytes at the ONH of POAG patients [37]. Here, our results showed that the expression of AQP9 was up-regulated with that of GFAP. This suggests that the IOP elevation may be related to glial activation since astrocytes express both proteins. Xue et al. reported that elevated IOP induced Müller glial cell activation manifested by increased GFAP expression and suggested the metabolic change of cells in response to the degenerative changes of their neighbouring ganglion cells [41,42]. Therefore the changes of water channel expression may be compensatory to the damage caused by elevated IOP. The lack of selective AQP inhibitors may prevent an evaluation of whether AQP9 inhibition is harmful in glaucomatous conditions.

AQP4 is another important water channel in the retina and is expressed by astrocytes and Müller cells. Unlike AQP9, AQP4 has exceptionally high water permeability and may serve different functions. Figure 4 shows the increased expression of AQP4 and AQP9 in the region of the optic nerve from the eye of rat exposed to experimentally elevated IOP treatment. Figure 5 shows the increased expression of AQP4 in the retina after the treatment by Western blotting. The smaller isoform of AQP4 showed a decreased expression whereas the higher molecular mass isoform (ubiquitinated forms) had increased expression [43]. Both results were concluded from five of seven rats (71%). In addition, rats with greater elevated IOP showed a more profound effect than the ones with less elevated IOP.

Previously, it has been reported that brain ischaemia with the breakdown of the BBB may cause enormous water entry, which down-regulated the expression of AQP4 at the early onset and up-regulated it thereafter [44]. The expression of AQP9 was noticeable after brain ischaemia, not during normal conditions [45]. Badaut et al. reported that transient ischaemia in the mouse brain is induced by arterial damage caused up-regulation of AQP9.
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**Figure 4** Expression of immunoreactive AQP9 (red) and AQP4 (green) in the optic nerve of rats exposed to experimentally elevated IOP

The contralateral (control) eyes showed modest AQP9 and AQP4 expression (B–D). In contrast, AQP9 and AQP4 labelling at the optic nerve was consistently more intense in eyes exposed to elevated IOP compared to control eyes (F–H). (A) and (E) are DIC images, (B) and (F) represent AQP9 labelling, (C) and (G) represent AQP4 labelling and (D) and (H) represent merged images of AQP9 and AQP4 labelling (yellow). Scale bars represents 50 μm.

**Figure 5** Elevation of IOP affected AQP4 isoforms differently

Elevated IOP appears to lower the expression of the smaller AQP isoform (∼30 kDa) while increasing the levels of the higher molecular weight AQP4 isoforms (∼37, 87 and 100 kDa). The higher molecular weight isoforms are ubiquitinated AQP4. Membranes were stripped, and then re-probed with anti-α-tubulin for normalization (C: control eye and T: elevated IOP eye).

**CONCLUSION**

The AQPs are water channel proteins that provide the key route for water movement across plasma membranes. AQP9 possesses common features of a water channel, but is also permeable to lactate and a wide selection of non-charged solutes, such as β-hydroxybutyrate, glycerol, carbamides, purines (adenine), pyrimidines (uracil), urea, mannitol and sorbitol, but impermeable to cyclic sugars [46]. In this study, an animal model of glaucoma was used to characterize the expression of AQP9 in the region of optic nerve and retina. The expression levels of AQP9 mRNA and protein were up-regulated in the retina. Also, the increased expression of immunoreactive AQP9 in the region of the optic nerve was detected. Such observation supports the theory that the solutes transported by AQP9 may be beneficial to the neurons.

**AUTHOR CONTRIBUTION**

All authors contributed to the study design, and data interpretation and analysis. Ming-Hui Yang conducted experiments, analysed data and wrote the paper. Adnan Dibas designed and conducted experiments. Yu-Chang Tyan analysed data and wrote/revised the paper.

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