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High-salt loading exacerbates increased retinal content of aquaporins AQP1 and AQP4 in rats with diabetic retinopathy

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ABSTRACT

In the neural retina, glial cells control formation of ionic gradients by mediating transmembrane water fluxes through aquaporin (AQP) water channels. Retinal content and immunolocalization of two water channels, AQP1 and AQP4, in the diabetic rat retinas during high-salt loading were examined in this study. Diabetes was induced by an intraperitoneal injection of streptozotocin. Diabetic and control animals were observed after varying lengths of exposure to normal- and high-salt conditions. Ultrathin sections of retinal tissue, stained with uranyl acetate and lead citrate, were photographed using a transmission electron microscope (TEM). Retinal wholemounts were immunostained with AQP1 and AQP4 antibody to detect the immunolocalization changes by confocal microscopy. AQP1 and AQP4 content were evaluated by Western blot analysis. In the retinas of high-salt loading diabetic animals, obviously increased intracellular edema was observed by TEM in ganglion cells and mitochondrial swelling was observed in glial cells. Immunolocalization of AQP1 increased from the posterior to peripheral retina. Western blot results indicated that a high-salt diet may cause increased retinal content of AQP4 and may exacerbate increased retinal content of AQP1 caused by diabetic retinopathy. High-salt loading may increase neural retinal edema in rats with diabetic retinopathy, and altered glial cell mediated water transport via AQP channels in the retina may play an important role in the neural retinal edema formation and resolution.

1. Introduction

The presence of retinal edema is a major cause of impaired vision in patients with diabetic retinopathy (DR) (Bresnick, 1983). Normally, fluid absorption from the inner retinal tissue occurs by transcellular water transport facilitated by aquaporin (AQP) water channels in glial cells (Nagelhus et al., 1998). In the brain, it has been shown that the development of edema is correlated with increased retinal content of water channels in glial cells. Increased AQP1 and/or AQP4 in reactive glial cells has been observed in various neurological disorders distinguished by edema, such as ischemia, trauma, brain tumors (Amiry-Moghaddam et al., 2004; Iandiev et al., 2006; Aoki et al., 2003; Badaut et al., 2003; Saadoun et al., 2002a,b), and also in DR (Iandiev et al., 2007).

In rodent retina, AQP1 and AQP4 are the most ubiquitous water channels. AQP1 is found in a subpopulation of amacrine cells and in photoreceptor cells, whereas AQP4 is expressed by glial cells, primarily Müller cells, and astrocytes (Iandiev et al., 2005; Kim et al., 1998, 2002; Nagelhus et al., 1998). AQP4 in Müller cells control osmotic balance in normal retina (Verkman et al., 2008). However, transient ischemia and diabetes cause an increase in immunoreactive AQP1 in retinal glial cells (Iandiev et al., 2006, 2007).

Metabolic and hemodynamic factors have been found to be causative in the pathogenesis of DR (Wilkinson-Berka and Fletcher, 2004). Indeed, hypertension has been identified as a major independent risk factor for the development and progression of DR in people with diabetes (UK Prospective Diabetes Study Group; Klein et al., 1998). Although hypertension often results from excessive sodium ingestion, the development of DR has not been directly linked to a high-salt diet.

High-salt loading disturbs ion balance and osmohomeostasis in cells (Thijssen et al., 2008). In animal models, dietary sodium intake has been causatively associated with damage of target organs, including those involved in the renal and cardiovascular systems, through mechanisms independent of blood pressure (Sanders, 2004; Sanders et al., 2005; Suzuki et al., 2004; Yu et al., 1998) (Yu et al., 1998; Du Cailar et al., 2004; Maeda et al., 2007). Although diabetic patients are often advised to decrease salt intake as

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a method of preventing the development of diabetic complications, the relationship between a high-salt diet and DR has not been isolated for objective study. In our previous study, we found that the occurrence of retinal disorders associated with a high-salt diet was linked to changes in glial cell AQP content (Qin et al., 2009). This study will examine the effect of a high-salt diet, independent of hypertension, on the occurrence of edema and glial cell AQP content in diabetic retinas.

2. Material and methods

2.1. Subjects

Sixty 6-week old male Wistar rats, each weighing approximately 150 g, were handled in accordance with the European Communities Council Directive 86/609/EEC, and the experimental protocol was approved by the local authorities. Diabetes was induced by an intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO, 65 mg/kg) diluted in 0.8 ml of 0.03 M citrate buffer (pH 4.7). Three days after administering the injection, samples of blood obtained from the tail vein of each rat were assayed to determine glucose concentration. Rats with blood glucose concentrations in excess of 16.7 mmol/L were diagnosed with diabetes. Systolic blood pressure was measured in conscious animals, via tail cuff plethysmography (BP Monitor for Rats and Mice Model MK-2000; Muramachi Kikai Co. Ltd., Tokyo, Japan). Rats with systolic blood pressures less than 120 mmHg were included in the study.

All animals had unrestricted access to tap water and food, containing varying concentrations of sodium chloride (NaCl), and were maintained in an air-conditioned room on a 12 h light:12 h dark cycle. Body weight, blood glucose concentration, and systolic blood pressure were monitored at 2 weeks intervals.

Diabetic and control animals were investigated after varying lengths of exposure to normal- and high-salt conditions. All animals were randomly divided into 12 groups. The treatments included low-salt non-diabetic control (LSNDC), low-salt diabetic (LSD), high-salt non-diabetic control (HSNDC), and high-salt diabetic (HSD). Food consumed by low-salt diet treatment groups contained 0.5% NaCl, and food consumed by high-salt diet treatment groups contained 8% NaCl. In each treatment, 3 groups ($n = 6$ each) were sacrificed after different lengths of exposure to the particular treatment (4, 8, and 12 weeks). The animals were euthanized with carbon dioxide, and the retinas were removed immediately.

2.2. Transmission electron microscopy (TEM)

Retina tissues were initially fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), then in 1.0% OsO4. They were dehydrated in a progressive ethanol and acetone solution, embedded in Epon812, sectioned with an LKB ultramicrotome, and stained with uranyl acetate followed by lead citrate. Prepared tissue samples were observed with a transmission electron microscope (Philips Model CM120; Holland).

2.3. Immunohistochemistry analysis

Retina tissues were fixed in 4% paraformaldehyde for 2 h. Subsequent immersion of the tissues in 10% bovine serum albumin for 2 h resulted in blocking of non-specific binding sites and permeabilization of retinal wholemounts. Wholemounts were incubated with primary antibodies for 72 h at 4 °C, washed in 1% bovine serum albumin, and incubated with secondary antibodies for 4 h at room temperature. Control wholemounts were not incubated with primary antibodies. The antibodies included mouse/rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Thermo/Abcom), mouse anti-glutamine synthetase (GS; 1:500; Chemicon), rabbit anti-AQP1 (1:200; Santa Cruz), mouse anti-AQP4 (1:500; Abcom), Cy3 conjugated goat anti-mouse IgG (1:400; Sigma), Alex 647-coupled goat anti-rabbit IgG (1:200; Molecular Probe). Blood vessels were stained with biotinylated Griffonia simplicifolia isolectin B4 (1:50; Sigma).

Retinas were divided into the posterior area (1 mm from the papilla), midperipheral area (1–2 mm from the papilla) and peripheral area (2–3 mm from the papilla), according to the previous protocol (Qin et al., 2006). A confocal laser scanning microscope TCS SP2 (Leica, Heidelberg, Germany) was used to observe and photograph samples.

2.4. Western blot analysis

Whole retinal samples were sonicated in radioimmunoprecipitation buffer containing various protease inhibitors (Upstate Biotechnology, Lake Placid, NY) and were centrifuged at 16,000 $\times$ g and 4 °C for 15 min to collect the supernatants. One retina from each of the 3 animals in each group were pooled for the sample.

After protein concentrations were determined by the Bradford protein assay kit (BioRad, Hercules, CA), equal 15 μg aliquots of retinal protein samples were applied to a 12% gradient sodium dodecylsulfate polyacrylamide gel (BioRad, Hercules, CA) and were separated by electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 10% nonfat dry milk for 1 h at room temperature. Retinal membranes were incubated with anti-AQP1 (1:500; Santa Cruz), anti-AQP4 (1:500; Abcom), anti-GFAP (1:500; Thermo) or anti-β-actin (1:2000; Santa Cruz; loading control) antibodies for12 h at 4 °C. Membranes were then incubated with the peroxidase-linked anti-mouse/rabbit IgG antibody (1:5000; Jackson) for 1 h at room temperature. Chemiluminescence signals were developed (ECL Western Blotting Substrate, Pierce, Rockford, IL) and were detected with a digital imaging system (IS4000R; Kodak, New Haven, CT). Care was taken to ensure that the intensities of detected bands were within the linear range of the camera and that no pixels were saturated. Protein band intensities were determined with Image J 1.32j software. Western blots were repeated three to five times and qualitatively similar results were obtained. Equal loading and transfer was ensured by reprobing the membranes for β-actin.

2.5. Statistical analysis of Western blot results

Protein band densities were measured in arbitrary densitometric units, and AQP1/actin, AQP4/actin and GFAP/actin ratios were calculated. One-way ANOVA followed by Fisher's protected least-significant difference test was used to assess the significance of differences between the treatment groups. The band density ratios of the HSNDC and LSD treatment groups with different lengths of exposure to treatment were compared to that of the LSNDC 8-week treatment group. Band density ratios of the HSD groups with different lengths of exposure were compared to those in the corresponding HSNDC and LSD groups. In the statistical analysis, p-values ≤ 0.05 were considered significant. Asterisk (*) on the columns indicated that band density ratio between the LSNDC treatment group and that of both the HSNDC and LSD treatment groups were significantly different. Asterisk (*) above the column indicates that band density ratios of the HSD groups were significantly different from those of the HSNDC and LSD treatment groups. *indicates p-values ≤ 0.05, ** indicates p-values ≤ 0.01.