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Intracerebral VEGF injection highly upregulates AQP4 mRNA and protein in the perivascular space and glia limitans externa

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Abstract

Stroke is the third leading cause of death and the leading cause of adult disability in the industrialized nations. One of the consequences of stroke is blood–brain barrier (BBB) leakage and subsequent edema, which is one of the causes of mortality in this pathology. Aquaporin-4 (AQP4) is the most abundant water channel in the brain. Studies in AQP4 knock-out mice have shown a prominent role of this water channel in edema development and resolution after ischemia. Here we have studied changes in AQP4 mRNA and protein expression in response to vascular endothelial growth factor (VEGF), a potent angiogenic factor. VEGF administration highly upregulated AQP4 mRNA and protein in the ventral midbrain. Perfusion of the animals with FITC-albumin prior to sacrifice demonstrated localization of AQP4 protein in close proximity to the VEGF-induced new blood vessels. Expression levels of AQP4 mRNA were maximum 7 days after VEGF injection whereas our previous report showed that BBB leakage is resolved at this time point. Therefore, we speculate a positive role of AQP4 in edema resolution, which may partially explain the previously reported beneficial effects of delayed VEGF administration in ischemic rats. Our results provide new insights into the molecular changes in the edematous brain and may help in future therapeutical directions.
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Keywords: Blood–brain barrier; FITC-albumin; Substantia nigra; Ventral mesencephalon

The aquaporins (AQPs) are a family of water-selective channel proteins which are present in many kinds of tissues (Venero et al., 2001; Agre et al., 2002; Papadopoulos et al., 2002; Verkman, 2002; Amiry-Moghaddam and Ottersen, 2003). AQPs mediate the efficient movement of water across the membrane. Eleven isoforms of AQPs, AQP0–AQP10, have been identified in mammalian cells to date, from which AQP4 is the most abundant water channel in the CNS (Jung et al., 1994). The highly polarized location of AQP4 on the membrane of astrocytic foot processes opposed to the brain capillaries, pia, and ependymal epithelium, points to its critical function in water transport across the blood–brain barrier (BBB) and brain–cerebrospinal fluid interfaces (Nielsen et al., 1997; Papadopoulos and Verkman, 2007).

In fact, analysis of AQP4-knockout mice has shown the connection between AQP4 expression changes and accumulation of water within the brain, i.e. edema (Manley et al., 2000; Papadopoulos et al., 2004; Venero et al., 2004; Papadopoulos and Verkman, 2007). Several studies have also unveiled a crucial role for AQP4 expression in response to brain edema formation after traumatic and ischemic insults (Vizuete et al., 1999; Vajda et al., 2000; Ke et al., 2001; Sun et al., 2003; Nesic et al., 2006). Moreover, enhanced AQP4 expression has been reported in a wide spectrum of human neuropathological conditions, including ischemia, trauma, brain tumours and multiple sclerosis (Saadoun et al., 2002; Aoki et al., 2003; Aoki-Yoshino et al., 2005). However, the significance of these changes is far to be understood. Therefore, it is of high interest to study regulation of AQP4 given the prominent role of this water channel in different pathological conditions in the CNS, mainly in injury paradigms where BBB is compromised, which are associated to vasogenic edema.

Abbreviations: AQP4, aquaporin-4; BBB, blood–brain barrier; IR, immunoreactivity; PD, Parkinson's disease; SN, substantia nigra; VEGF, vascular endothelial growth factor.

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Vascular endothelial growth factor (VEGF) is the most potent inducer of BBB permeability and disruption (Ayata and Ropper, 2002; Venero et al., 2004). In a previous study we demonstrated that in vivo VEGF administration into the SN induces a cascade of events that typically resemble histopathological features of Parkinson's disease (PD) exemplified by a significant and selective loss of dopaminergic neurons in this region (Rite et al., 2007). Since the main key feature that was observed in response to VEGF administration was disruption of the BBB, we concluded that VEGF-induced BBB breakdown may be the causative factor for the degeneration of the nigral dopaminergic neurons observed in this model. Taking into account the well-known role of AQP4 in brain–blood homeostasis, we have studied expression of AQP4 mRNA and protein after intranigral VEGF injection.

1. Experimental procedures

1.1. Animals and surgery

Male Wistar rats (230–250 g) housed in our laboratory were used for these studies. For stereotaxic injection of VEGF into the left SN, the rats were anesthetized with 400 mg/kg of chloral hydrate and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) to conform to the brain atlas of Paxinos and Watson (1986). VEGF (Sigma, St. Louis, MO, USA) was dissolved in Monastral Blue inert tracer (Sigma, 1% in phosphate buffer saline, PBS). The injection needle was lowered through a drilled hole 5.5 mm posterior, 1.8 mm lateral and 8.3 mm ventral to bregma; and 1 μg of VEGF was injected in a final volume of 2 μl. As control, VEGF vehicle (1% Monastral Blue-PBS) was injected into the right SN.

Animals used for in situ hybridization ($n = 12$) were sacrificed by decapitation 1, 4 and 7 days after surgery ($n = 4$ each group). Brains were immediately removed and then frozen in isopentane at −15 °C.

To study both AQP4 protein expression and BBB leakage, three animals were intracardially infused 4 days after surgery with 10 ml FITC-albumin (Sigma, 5 mg/ml) according to the method of Cavaglia et al. (2001). Within 2 min, the brains were removed and immediately immersed into formalin. The following day, the fixative was replaced with 30% sucrose.

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals and approved by the Scientific Committee of the University of Seville. All efforts were made to minimize animal suffering and to reduce the number of animals used.

1.2. AQP4 in situ hybridization

The pBluescript SK plasmid containing the cDNA sequence for AQP4 [33] was purchased from the American Type Culture Collection (Rockville, MD, USA). Antisense riboprobe contains a 394-nucleotide fragment from the 5′ end of the rat AQP4 gene, which corresponds to nucleotides 1201–1595 of the rat AQP4 sequence. Sense and antisense riboprobes were synthetized as described in Tomas-Camardiel et al. (2004) and transcribed in the presence of [^{35}S]-UTP (1300 Ci/mmol; Amersham, Sweden).

In situ hybridization histochemistry was performed as described in Rite et al. (2007). Hybridization was done for 3 h at 50 °C in a buffer containing 50% deionized formamide, 10% dextran sulphate, 1× Denhardt solution, 2× SCC, 0.1% sodium pyrophosphate, 100 μg/ml tRNA, 100 μg/ml denaturated salmon sperm DNA and the [35S]riboprobe. Following incubation and to eliminate unspecific bindings, sections were treated with RNase (20 μg/ml RNase A in 0.5 M NaCl, 0.01 M Tris–HCl, 0.001 M EDTA, pH 8.0) and washed in a series of SSC solutions. Autoradiograms were generated by opposing the labelled tissue to β_{max} Hyperfilm (Kodak, USA) for 2 weeks.

Following dry-film autoradiography, sections were processed for emulsion autoradiography using Amersham LM1 emulsion (diluted 1:1 with water) and exposed in the dark at 4 °C for 2 months. Slides were developed in D-19 (Kodak) and counterstained with cresyl violet (Sigma).

1.3. In situ hybridization data analysis

For quantification of AQP4 mRNA expression, dry-film autoradiographs were scanned and analyzed by densitometry using the computer program analySIS® (Soft Imaging System, Germany). The region of interest was delineated and its optical density measured based upon calibrated gray scale densities resulting in relative values. Delineation in substantia nigra included the pars compacta and pars reticulata of this brain area. Measurements were performed in ipsilateral and contralateral regions to detect the VEGF-induced changes in optical densities. Representative nigral tissue sections from the same rostrocaudal level from three animals for each time point – 1, 4 and 7 days – were processed for such analysis. To detect changes at the cellular level, quantification of silver grain densities over individual cell nuclei was carried out using a Computer Enhanced Video Densitometer (analySIS®, Soft Imaging System, Germany). The system was calibrated by measuring increasing silver grain densities. Labelling was considered specific when grain accumulation over cells exceeded five-times the background value. The area with high grain density over an individual cell nuclei was delineated and the number of grains within this field was counted by means of the computerized image analysis system with a 40× magnification lens.

1.4. Detection of AQP4 by fluorescence immunohistochemistry

Sections were rehydrated in TBS for 10 min, and blocked with TBS containing 1% goat serum for 1 h. Slides were, then, incubated with AQP4 antibody (Chemicon, 1:100 in blocking solution containing 0.25% Triton-X-100) overnight at 4 °C. Labelling was visualized using anti-rabbit Cy3-conjugated antibody (Vector, 1:500). To detect any unspecific labelling, controls were performed incubating sections only with the secondary antibody.

1.5. Quantification of blood vessel density and AQP4 protein expression

Density of blood vessels was assessed by quantifying the percentage of the fluorescent positive area after perfusion with FITC-albumin in the ventral midbrain to the total area of the substantia nigra using the computer program analySIS®. Image contrast was adjusted such that the labelled vessels were above the software analysis detection threshold, whereas the parenchyma remained below threshold. AQP4 protein expression in SN was measured in a similar way in order to allow direct comparisons between the angiogenic effect of VEGF and AQP4 protein expression. AQP4 protein expression in glia limitans externa was measured following a different approach to that employed for the perivascular compartment. Representative sections from each animal were photographed at 40× magnification under identical bright and contrast conditions. The region of interest was delineated and the area displaying AQP4 measured. The optical density was then measured based on calibrated gray scale densities resulting in relative values. An index of AQP4 immunoreactivity was calculated as the product of optical density and area measured.

1.6. Statistical analysis

Results are typically expressed as mean ± S.D. Means were compared. Results were analyzed by one-way ANOVA. The Tukey's test was used for post hoc multiple range comparisons.

2. Results

2.1. Expression of AQP4 mRNA in the ventral mesencephalon in response to VEGF

Expression of AQP4 mRNA was first analyzed at 1, 4 and 7 days after the injection of VEGF in the SN from dry-film autoradiographs. VEGF highly induced AQP4 mRNA levels in

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