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DIFFERENTIAL EXPRESSIONS OF AQUAPORIN SUBTYPES IN ASTROGLIA IN THE HIPPOCAMPUS OF CHRONIC EPILEPTIC RATS

J.-E. KIM,[a,b] H. J. RYU,[a,b] S.-I. YEO,[a,b] C. H. SEO,[c] B. C. LEE,[d] I.-G. CHOI,[d] D.-S. KIM[e] AND T.-C. KANG[a,b]*

[a]*Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, South Korea*

[b]*Institute of Epilepsy Research, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, South Korea*

[c]*Department of Rehabilitation Medicine, Hangang Sacred Heart Hospital, Hallym University, Seoul 150-719, South Korea*

[d]*Department of Psychiatry, Hangang Sacred Heart Hospital, Hallym University, Seoul 150-719, South Korea*

[e]*Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan, Chungcheongnam-Do 330-090, South Korea*

Abstract—In order to elucidate the roles of aquaporins (AQPs) in astroglial responses, we investigated AQP expressions in the experimental epileptic hippocampus. In control animals, AQP1 protein expression was restricted to the ventricular-facing surface of the choroid plexus. AQP4 was expressed in astrocyte foot processes near blood vessels and in ependymal and pial surfaces in contact with cerebrospinal fluid. AQP9 protein has been detected in cells lining the cerebral ventricles, and in astrocytes. Six to eight weeks after status epilepticus (SE), AQP1 expression was mainly, but not all, detected in vacuolized astrocytes, which were localized in the stratum radiatum of the CA1 region. AQP4 was negligible in vacuolized CA1 astrocytes, although AQP4 immunoreactivity in non-vacuolized astrocytes was increased as compared to control level. AQP9 expression was shown to be mainly induced in non-vacuolized CA1 astrocytes. Therefore, our findings suggest that AQP subunits may play differential roles in various astroglial responses (including astroglial swelling and astroglial loss) in the chronic epileptic hippocampus.

Key words: aquaporin 1, aquaporin 4, aquaporin 9, astrocytes, epilepsy, hippocampus.

Aquaporins (AQPs) are water channels that provide the major route for water movement across plasma membranes in a variety of tissues including the brain (Agre et al., 2002; Verkman, 2002; Manley et al., 2000; Badaut et al., 2002; Amiry-Moghaddam and Ottersen, 2003). In normal rat brain, six AQPs subtypes have been described: AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9 (Badaut et al., 2001; Elkjaer et al., 2000; Nielsen et al., 1997; Venero et al., 1999; Yamamoto et al., 2001). Among them, AQP1, AQP4 and AQP9 are identified by their protein expression levels and localizations in the rat brain. AQP1 protein expression is restricted to the ventricular-facing surface of the choroid plexus. AQP4 is expressed in astrocyte foot processes near blood vessels in rat and in ependymal and pial surfaces in contact with cerebrospinal fluid. AQP9 protein has been detected in cells lining the cerebral ventricles, including ependymal cells and tanycytes, and astrocytes (for review, Badaut et al., 2002).

Astrocytes play a role in maintenance of homeostasis in the brain by regulating local ion concentrations, pH and clearance of neurotransmitters released into the synaptic cleft. Furthermore, astrocytes release many neuroactive substances, such as chemical transmitters, cytokines, neuropeptides and growth factors (Anderson and Swanson, 2000; Ransom et al., 2003). Reactive astrogliosis represents high glial fibrillary acidic protein (GFAP) and their cell bodies hypertrophy, and begin to proliferate, migrate and form glial scars, which is frequently encountered in association with temporal lobe epilepsy in humans and with drug- or kindling-induced seizures in animal models (Bordey and Sontheimer, 1998; Mathern et al., 1998). This reactive astrogliosis is considered as a consequent healing process that produces pathological effects by interfering with the functions of residual neuronal circuits (Represa et al., 1995), or as a compensatory response, that provides trophic factor to survived neuronal populations (Ridet et al., 1997). Interestingly, Revuelta et al. (2005) reported astroglial death in the CA1 region and the amygdalar complex after kainic acid administration. We also found TUNEL positive astroglial death in the rat dentate gyrus after pilocarpine-induced status epilepticus (SE) (Kang et al., 2006) and TUNEL negative astroglial hypertrophy and vacuolization in the stratum radiatum of the CA1 region (Kim et al., 2008a), which is considered an early stage of necrosis (Deloncle et al., 2001; Sugawara et al., 2002; Tomimoto et al., 1997). Unlike that observed in the dentate gyrus and entorhinal cortex, furthermore, conventional anti-epileptic drugs prevented delayed necrotic astroglial degeneration in the CA1 region. Thus, these findings reveal that CA1 astroglial damage may be a consequence of prolonged seizure. Although AQPs are supposed to play a role in astroglial hypertrophy/vacuolization (for review, Badaut et al., 2002), the underlying molecules involved in delayed necrotic astroglial degeneration are still unclear. Therefore, the present study was designed to elucidate the relationship between astroglial responses and AQPs in the experimental epileptic hippocampus.

*Correspondence to: T.-C. Kang, Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon, Kangwon-do 200-702, South Korea. Tel: +82-33-248-2524; fax: +82-33-256-1614.
E-mail address: tckang@hallym.ac.kr (T.-C. Kang).

Abbreviations: AQP, aquaporin; GFAP, glial fibrillary acidic protein; GFAP$^+$, glial fibrillary acidic protein immunoreactive; PBS, phosphate-buffered saline; SD, Sprague–Dawley; SE, status epilepticus.

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EXPERIMENTAL PROCEDURES

Experimental animals

This study utilized the progeny of Sprague–Dawley (SD) rats obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (22±2 °C, 55±5% and a 12-h light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, 1996). We have made all efforts to minimize the number of animals used and their suffering.

Seizure induction and drug treatments

Male SD rats (9 weeks old, $n=20$) were treated with pilocarpine (Sigma-Aldrich Co., St. Louis, MO, USA; 380 mg/kg i.p.) at 20 min after atropine methylbromide (Sigma-Aldrich Co., St. Louis, MO, USA; 5 mg/kg i.p.). Among pilocarpine-treated rats, 17 rats showed acute behavioral features of SE (including akinesia, facial automatisms, limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements and falling). Diazepam (10 mg/kg i.p.; Valium, Hoffman la Roche, Neuilly sur-Seine, France) was administered 2 h after onset of SE and repeated, as needed. Rest animals (non-experienced SE animals, $n=3$) showed only acute seizure behaviors during 10–30 min. Rats that did not experience SE were used as controls, acute or brief pilocarpine-induced seizure could not result in neuropathological changes in the rat brain (Kim et al., 2009; Bower and Buckmaster, 2008). Indeed, we could not observe changes in AQP expression in these animals (data not shown). In addition, 30 min before pilocarpine treatment, diazepam (10 mg/kg i.p.) was given to some animals. Diazepam pretreatment completely prevented SE. Diazepam-pretreated animals used immunohistochemical studies at designated time courses (3 days 1 week and 5 weeks after SE, $n=3$, respectively). Age-matched animals ($n=8$) were also used as controls. One week after SE, rats were observed 3–4 h a day in the vivarium for general behavior and occurrence of spontaneous seizures. The onset of spontaneous complex partial seizure occurrence was approximately 4 weeks after SE. On average, these animals developed two seizures/day.

Tissue processing and immunohistochemistry

At designated time courses, animals were anesthetized (urethane, 1.5 g/kg, i.p.; Sigma-Aldrich Co., St. Louis, MO, USA) and perfused transcardially with phosphate-buffered saline (PBS, Sigma-Aldrich Co., St. Louis, MO, USA) followed by 4% paraformaldehyde (Sigma, MO, USA) in 0.1 M PB (pH 7.4; Sigma-Aldrich Co., St. Louis, MO, USA). The brains were removed, and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the tissues were frozen and sectioned with a cryostat at 30 μm and consecutive sections were collected in six-well plates containing PBS. The sections were first incubated with 3% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS for 30 min at room temperature. Sections were then incubated in primary antibodies (listed below) in PBS containing 0.3% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO, USA) overnight at room temperature: rabbit anti-AQP1 (Abcam, Cambridge, UK, diluted 1:200), AQP4 (Chemicon, CA, USA, diluted 1:200) or AQP9 IgG (LifeSpan, Seattle, WA, USA, diluted 1:200). The sections were washed three times for 10 min with PBS, incubated sequentially, in biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector, Burlingame, CA, USA) and ABC complex (Vector, Burlingame, CA, USA), diluted 1:200 in the same solution as the primary antiserum. Between incubations, the tissues were washed with PBS three times for 10 min each. The sections were visualized with 3,3′-diaminobenzidine (DAB, Sigma-Aldrich Co., St. Louis, MO, USA) in 0.1 M Tris buffer and mounted on gelatin-coated slides. The immunoreactions were observed under the Axiophot microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany). All images were captured using an Axiocam HRc camera and Axio Vision 3.1 software. Double immunofluorescent staining for AQP1, AQP4 or AQP9/GFAP was also performed. Brain tissues were incubated in mixture of rabbit anti-AQP1, AQP4 or AQP9 IgG (diluted 1:50)/mouse anti-GFAP IgG (diluted 1:100) overnight at room temperature. After washing three times for 10 min with PBS, sections were also incubated in a mixture of FITC- and Cy3-conjugated secondary antisera (1:200, Amersham, PA, USA) for 1 h at room temperature. Sections were mounted in Vectashield mounting medium (Vector, Burlingame, CA, USA). All images were captured using an Axiocam HRc camera and Axio Vision 3.1 software. In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of the primary antibody (for GFAP) or a pre-absorption test was performed with control peptide (for AQPs). The control for immunohistochemistry resulted in the absence of immunoreactivity in any structure (data not shown). All experimental groups in the present study were included in each immunochemistry and were therefore processed under the same conditions.

Quantification of data and statistical analysis

For quantification of GFAP/AQPs double immunofluorescence, we have performed the cell count. GFAP and AQP immunofluorescent images (10 sections/rat) were captured in the same region (500×500 μm). Images were sampled from at least five different points within each hippocampal section. Thereafter, the number of GFAP positive cells that are each AQP positive was actually counted within the sampled images. All immunoreactive cells were counted regardless the intensity of labeling. The number of vacuolized GFAP positive cells was counted by the same method. The diameter of vacuoles in astrocytes was also measured by Axio Vision 3.1 software. Cell counts and the measurement of the diameter of vacuoles were performed by two different investigators who were blind to the classification of tissues. All data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine statistical significance. Bonferroni's test was used for post hoc comparisons. A P-value below 0.05 was considered statistically significant (Kim JE et al., 2008, 2009).

RESULTS

Coagulative necrosis of astrocytes in the CA1 region

Generally, vacuolization of cells may be considered as early stage of necrosis, because these cells often show necrotic features, such as eosinophilic cytoplasm, mitochondrial/nuclear membrane alterations, or TUNEL negativity (Deloncle et al., 2001; Sugawara et al., 2002). Similarly, astroglial hypertrophy and vacuolization are reported in various neurodegenerative diseases (Deloncle et al., 2001; Sugawara et al., 2002; Tomimoto et al., 1997). In our previous study (Kim DS et al., 2008), we had also reported that prolonged recurrent seizure resulted in TUNEL negative loss/vacuolization of CA1 astrocytes (presumably delayed necrotic astroglial damage). In the present study, at 6–8 weeks after SE we identified vacuolized astrocytes only in the stratum radiatum of the CA1 region by H & E staining. On the basis of the localization and nuclear size/shape, we could detect astrocytes on H-E-stained slides.