ARTICLE
Journal of Cellular Biochemistry 114:111–119 (2013)

Journal of Cellular Biochemistry

Hyperosmotic Induction of Aquaporin Expression in Rat Astrocytes Through a Different MAPK Pathway

Mei Yang,[1] Fei Gao,[2] Hui Liu,[1] Wei Hua Yu,[1] Fei Zhuo,[1] Guo Ping Qiu,[1] Jian Hua Ran,[1] and Shan Quan Sun[1*]

[1] *Institute of Neuroscience, Chongqing Medical University, Chongqing 400016, People's Republic of China*

[2] *Department of Urology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China*

ABSTRACT

Water homeostasis of the nervous system is important during neural signal transduction. Astrocytes are crucial in water transport in the central nervous system under both physiological and pathological conditions. To date, five aquaporins (AQP) have been found in rat brain astrocytes. Most studies have focused on AQP4 and AQP9, however, little is known about the expression of AQP3, -5, and -8 as well as their regulating mechanism in astrocytes. The expression patterns of AQP3, -5, and -8 in astrocytes exposed to hyperosmotic solutions were examined to clarify the roles of AQP3, -5, and -8 in astrocyte water movement. The expression of AQP4 and AQP9 under the same hyperosmotic conditions was also investigated. The AQP4 and AQP9 expressions continuously increased until 12 h after hyperosmotic solution exposure, whereas the AQP3, -5, and -8 expressions continued to increase until 6 h after hyperosmotic solution exposure. The different AQPs decreased at corresponding time points (24 h for AQP4 andAQP9; 12 h for AQP3, -5, and -8 after hyperosmotic solution exposure). The ERK inhibitor can attenuate the expression of AQP3, -5, and -8 after hyperosmotic solution exposure. The p38 inhibitor can inhibit the AQP4 and AQP9 expressions in cultured astrocytes. AQP expression is directly related to the extracellular hyperosmotic stimuli. Moreover, different AQPs can be regulated by a distinct MAPK signal transduction pathway. J. Cell. Biochem. 114: 111–119, 2013.

KEY WORDS: AQUAPORIN; ASTROCYTE; HYPEROSMOTIC; MAPK

The regulation of tissue water content and brain volume is critical to the normal functioning of the central nervous system. An astrocyte is a widespread cell in the brain that plays a central role in brain homeostasis, such as brain blood flow regulation, blood–brain barrier formation, brain ion homeostasis control, and synaptic plasticity modulation. Therefore, understanding the astrocytic water transport, especially under some pathological conditions, is essential.

Water has been proven to pass across cell membranes substantially faster than simple diffusion allows. Aquaporins (AQPs) are a family of membrane channel proteins that have been suggested to maintain the water homeostasis of the brain [Venero et al., 2001; Badaut et al., 2002; Papadopoulos et al., 2002]. The expression of some AQPs is sensitive to various factors, including brain injury, hypoxia and reoxygenation, edema, and some other experimental conditions [Yamamoto et al., 2001; Yoneda et al., 2001; Fu et al., 2007]. Osmotic stress possibly damages DNA and proteins, resulting in the impairment of cell function and induction of protection systems and repair processes [Dmitrieva and Burg, 2005]. These relatively nonspecific responses to cell damage are possibly an important aspect of cellular adaptation to osmotic stress. To date, five AQPs have been found in mature astrocytes, namely, AQP3, -4, -5, -8, and -9 [Yamamoto et al., 2001]. These five AQPs are distributed in the same cell type, which is the most

Disclosures: The authors declare that they have no conflict of interest associated with this study.

Fei Gao, Hui Liu, and Wei Hua Yu contributed equally to this study.

Grant sponsor: National Natural Science Fund of China; Grant number: 81000566; Grant sponsor: Excellent Program for Young Teacher of Chongqing Medical University; Grant sponsor: Ph.D. Programs Foundation of Ministry of Education of China; Grant number: 20105503120009.

*Correspondence to: Dr. Shan Quan Sun, Yixue Road 1, Chongqing, Chongqing 400016, People's Republic of China. E-mail: sunsq2151@yahoo.com

Manuscript Received: 7 November 2011; Manuscript Accepted: 26 July 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 6 August 2012

DOI 10.1002/jcb.24308 • © 2012 Wiley Periodicals, Inc.

abundant nerve cell (astrocyte), therefore, the possible role differences of these AQPs under stimuli in an astrocyte is interesting to study. Except for AQP4, the other four AQPs are also distributed in neurons. AQP3 and AQP9 can also transport glycerol or small molecules [Verkman, 2001; Badaut, 2012]. Although previous studies have proven that AQP4 and AQP9 were affected by hyperosmotic stress [Arima et al., 2003], little is known about the role of other AQPs under hyperosmotic conditions. In addition, previous studies focused on one content that made a solute hyperosmotic, which was not similar to clinical conditions. The concentration of all the materials in the blood is relatively increased when hyperosmotic dehydration occurs. Thus, a completely hyperosmotic culture medium was applied based on our previous study [Li and Sun, 2004]. Recent studies from this laboratory have documented the involvement of AQP3, -5, and -8 in brain edema [Yang et al., 2009]. These three AQPs are also possibly related to hyperosmotic stimulation, however, this hypothesis needs to be investigated.

The control of water flux and cell volume is critical in the brain. Moreover, solute transport regulation is critical because osmolality changes in extracellular fluids can affect neuronal cell function. Mitogen-activated protein kinases (MAPKs), specifically extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, are important intracellular signal transduction pathways activated in response to osmolality changes [Galcheva-Gargova et al., 1994; Schmidt and Schimmel, 1994; Matsuda et al., 1995; Burg et al., 1996]. Previous studies have reported that AQP4 and AQP9 expressions in astrocyte under hyperosmotic conditions are regulated by MAPKs [Arima et al., 2003]; however, the role of MAPKs in other AQP expressions of astrocyte under hyperosmotic stress is unclear. The present study aims to investigate AQP expressions in astrocytes under hyperosmotic stress, as well as the role of MAPKs in the AQP expressions.

MATERIALS AND METHODS

CELL CULTURE

Primary cortical astrocytes were prepared from the brains of postnatal day (0–1) rats using a previously described method [Kato et al., 1979]. Briefly, rat brain tissue was freed of meninges, dissected via trituration and vortexing, passed through sterile nylon sieves, placed in Dulbecco's modified Eagle medium (DMEM; Hyclone), and then supplemented with penicillin, streptomycin, and fetal bovine serum. The medium was replaced after 24 h and every third day thereafter. The cells were grown to 80% confluency, and were subsequently trypsinized and subcultured. Cultures consisted of at least 95% astrocytes, as determined by immunocytochemical examination with astrocyte anti-glial fibrillary acidic protein (GFAP). The third passage cells were used in the present study. All procedures were performed in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996) and were approved by the Animal Welfare Committee of Chongqing Medical University. Cells were randomly divided into two groups, namely, control group (282.5 mmol/L) and hyperosmotic group (320, 333, and 345 mmol/L). The cultures were incubated for certain periods (0, 1, 3, 6, 12, and 24 h) with or without hyperosmotic medium. Culture medium osmolality was measured using the freezing point depression method. All cells were incubated at 37°C in a 95% O_2/5% CO_2 humidified atmosphere. In the MAP kinase inhibitor (U0126, SB203580, SP600125) or activation studies (anisomysin or TPA), reagents were added in the specified concentration for a 1-h preincubation or siRNA for ERK and p38 was transfected at 2 days before the addition of an isotonic or hyperosmotic medium. Cells were then maintained in the medium for the duration of the experiment.

MTT REDUCTION TEST

Astrocyte viability was quantitatively assessed by the MTT reduction test. Astrocytes were incubated with 20 μL of MTT (5 mg/mL) at 37°C in culture medium for 4 h after exposure to hyperosmotic medium. Cultures were then washed and incubated in dimethyl sulfoxide (DMSO; 150 μl). Cell viability was evaluated by optical density, and read at 570 nm using an automatic universal microplate spectrophotometer (Type 3550; BioRad). Results are presented as the percentage of the control optical density measured in the control group.

ESTIMATION OF CELL VOLUME

The 10-Hz confocal x–y images of the same cell under both isotonic or hyperosmotic conditions were converted to a binary format using the ImageJ software. The comparative z-position was maintained and confirmed by the appropriation of the z-plane with the largest surface area, as well as cross-referencing fluorescence intensity from other areas of the selected image. The surface area was then calculated using the particle analysis software in ImageJ [Maric et al., 2001]. Each cell volume estimate was made from at least 10 independent cells, from at least three separate experimental determinations.

REVERSE TRANSCRIPTION REAL-TIME PCR

Total RNA from tissue specimens was isolated using Tissue/cell RNA Mini kit at the different time points. The cDNAs were generated from 1 μg of total RNA by superscript. RNase H^- reverse transcriptase with Oligo (dT) primer. Quantity of AQPs mRNA levels was done by real-time PCR using Taqman probe. The primers were given in Table I. The primer pairs were designed from rat AQPs mRNA sequences retrieved from GeneBank. The beta-actin primer sets were included as house-keeping control genes.

Reactions were carried out in 50 μl volumes consisting of Premix Ex Taq (2×) 25 μl, PCR forward primer (10 μm) 1 μl, PCR reverse primer (10 μm) 1 μl, Taqman probe (20 μm) 2 μl, Rox reference Dye (50×) 1 μl, cDNA 5 μl, and ddH_2O 15 μl (TaKaRa, China). Each run consisted of serial dilution (10×) of standard preparation and rat cDNA samples to generate a standard curve. In each reaction, 5 μl cDNA was amplified. The amplification program was as follows: preincubation at 95°C for 10 s, fast start polymerase action at 95°C for 5 s, followed by 60°C for 31 s. Taqman probe fluorescence was acquired at 60°C in each amplification cycle. Changes in AQPs mRNA expression were examined with ABI7000 Sequence Detection System (Perkin Elmer). A standard curve was used to extrapolate the copy number of target cDNA in rat astrocytes.

TABLE I. Gene-Specific Real-Time PCR Primers, TaqMan Probes, and Their Respective PCR Fragment Lengths

	Forward primer	Reverse primer	TaqMan-Probe	Length (bp)
β-actin	5′-CCCTGGCTCCTAGCACCAT-3′	5′-CACAGAGTACTTGCGCTCAGGA-3′	5′-FAAGATCAAGATCATTGCTCP-3′	186
AQP3	5′-TGCTGGGATTGTTTTTGGG-3′	5′-GCCGGAGACAACAAGCTCAT-3′	5′-FCTACTATGATGCAATCTGP-3′	73
AQP4	5′-CTGCAGTTATCAATGGGAACTGG-3′	5′-GCGCCTATGATTGGTCCAAC-3′	5′-FAAACCACTGGATATATTGP-3′	64
AQP5	5′-GCCACATCAATCCAGCCATT-3′	5′-GGAGCAGCGAGATCTGGTTT-3′	5′-FTCTGGCCCTCTTAATAGP-3′	60
AQP8	5′-GTTCATGCAGGCTCCAGAGAT-3′	5′-CCATTGGTTTCTCTGTGTCATTGTG-3′	5′-FCCACCACCTGCCAGGAP-3′	65
AQP9	5′-TCCCAGGCTCTTCACTGCA-3′	5′-ACCCACGACAGGTATCCACC-3′	5′-FTTGACCTCAACACAGTTGGP-3′	85

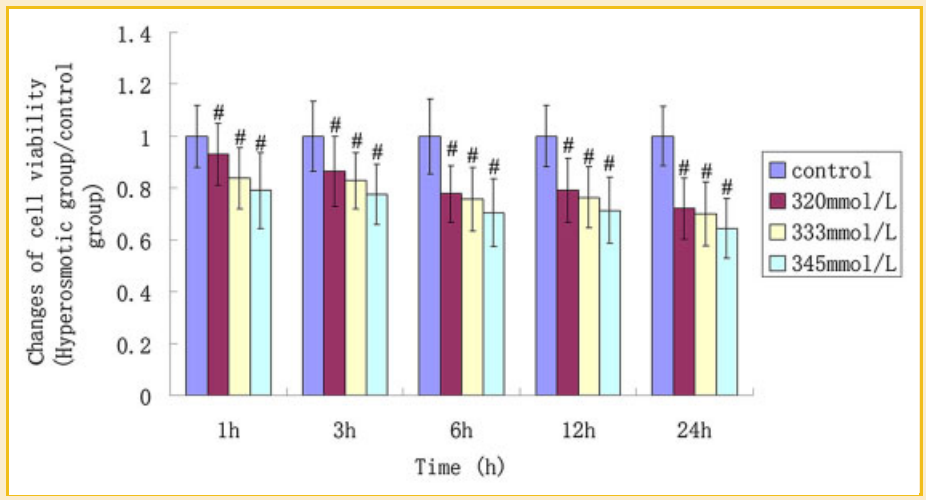

Fig. 1. Astrocyte viability after exposure to hyperosmotic medium was assessed by the MTT reduction rest. Data are expressed as the mean percent of the optical density measured in control group (#$P < 0.05$ vs. control). $n = 8$ cultures for each condition.

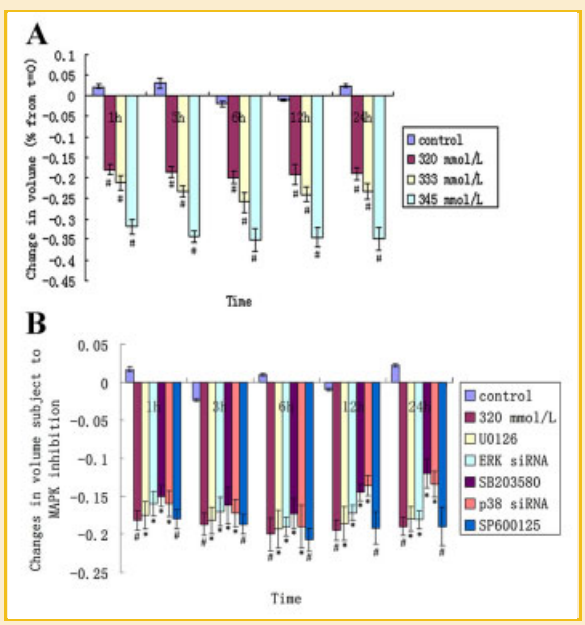

Fig. 2. Mean changes in cell volume in response to hyperosmotic stress. A: Changes of cell volume in different hyperosmotic stress. B: Astrocytes were incubated in 320 mmol/L hyperosmotic medium. Changes of cell volume subject to MAPK inhibitor or siRNA (#$P < 0.05$ vs. control; *$P < 0.05$ vs. control; and 320 mmol/L hypertonic group).

WESTERN BLOT ANALYSIS

The medium was aspirated and cells were washed in ice-cold phosphate-buffered saline at corresponding time points prior to scraping. Scraped cells were pelleted at 10,000*g* for 1 min at 4°C and resuspended in ice-cold homogenization buffer containing 50 mM ethylenediamine tetraacetic acid (EDTA), 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 mM phenylmethysufonyl fluoride (PMSF), and 200 KIE/ml aprotinin. The homogenates were then centrifuged at 10,000*g* for 20 min at 4°C The supernatant was collected and protein concentration was determined using a Bradford assay kit (Bio-Rad, Hercules). Samples were separated by 12% SDS–PAGE gels, and then transferred onto PVDF membranes. Blotted membranes were blocked with 5% skim milk, and subsequently incubated with primary antibodies against AQPs (1:500, rabbit anti-AQP; Alpha Diagnostic International), or antibodies against p38 (1:500, goat anti-p38; Santa Cruz), or antibodies against phosphorylated p38 (1:1,000, goat anti-pp38; Santa Cruz), or antibodies against ERK1/2 (1:1,000, rabbit anti-ERK1/2; Santa Cruz), or antibodies against phosphorylated ERK1/2(1:1,000, goat anti-pERK1/2; Santa Cruz), or antibodies against JNK(1:500, rabbit anti-JNK; Santa Cruz), or antibodies against phosphorylated JNK (1:1,000, goat anti-pJNK; Santa Cruz). Thereafter, the membranes were incubated with horse radish peroxidase-conjugated secondary antibodies (Santa Cruz). The optical densities of AQPs, MAPK, and beta-actin bands were quantitatively analyzed with gel densitometry (Bio-Rad). The results were expressed as AQP/beta-actin, or MAPK/beta-actin, or pMAPK/

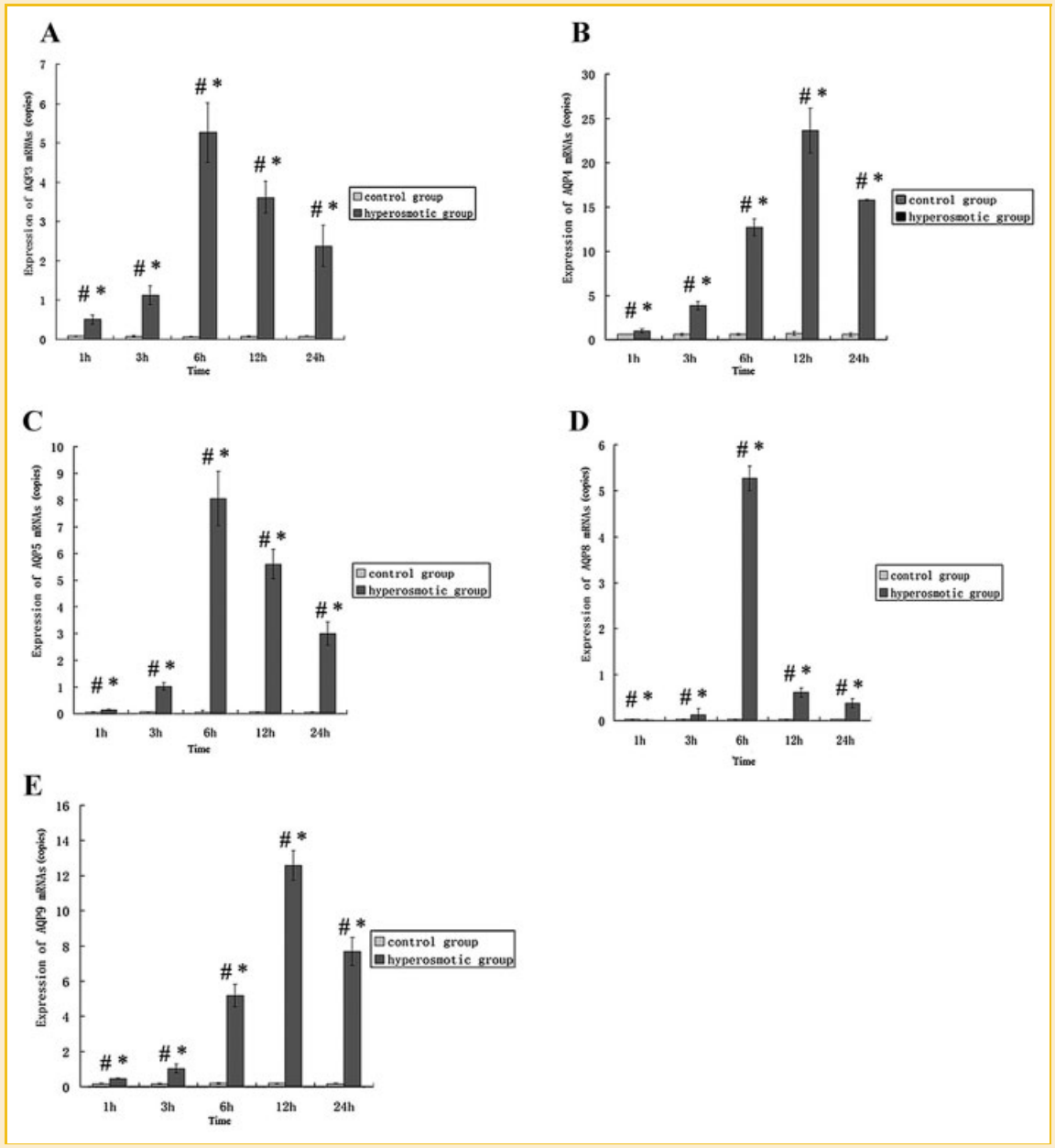

Fig. 3. Temporal changes of AQPs mRNA expression after exposure to hyperosmotic medium. Real-time PCR was used to detect mRNA levels in astrocytes at 1, 3, 6, 12, and 24 h after exposure to hyperosmotic medium. A, B, C, D, and E represent the mRNA levels of AQP3, AQP4, AQP5, AQP8, and AQP9, respectively (#$P < 0.05$ vs. control; *$P < 0.05$ vs. other point time in the same hypertonic group).

beta-actin. Each point was repeated in triplicate. The specific reaction was visualized by a chemiluminescent substrate (Pierce). Western bands were quantified by gel densitometry (Bio-Rad). The AQP protein band value was divided by the beta-actin value for the same sample, thus a protein:beta-actin ratio for each sample was obtained (each point was repeated in triplicate). Bands were normalized with the beta-actin loading control.

RNA INTERFERENCE

Cells were transfected with small-interfering RNA (siRNA) targeting rat ERK (Santa Cruz) and p38 or control siRNA (Cell Signaling Technology) using Lipofectamine 2000, as recommended by the manufacturer. Immunoblotting was performed after 48 h of transfection.

STATISTICAL ANALYSIS

All statistics were performed using the SPSS 11.0 software package (SPSS Inc., Chicago, IL). The level of AQPs protein and mRNA expression or MAPK protein expression for specimens were expressed as means ± SD. Differences between individual groups were first compared using analysis of variance (one-way ANOVA); thereafter, data were analyzed with LSD multiple comparisons post hoc testing. All reported *P*-values were two-sided, and a value of $P < 0.05$ was considered statistically significant.

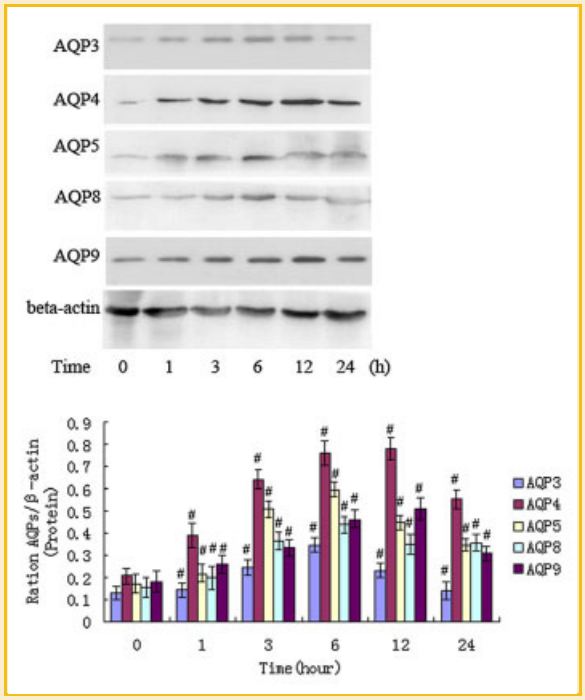

Fig. 4. Western blotting analysis of AQP protein levels in hyperosmotic medium. Western blotting analysis of AQPs protein levels (AQP3, AQP4, AQP5, AQP8, and AQP9) in astrocytes at 0, 1, 3, 6, 12, and 24 h after exposure to hyperosmotic medium (#$P < 0.05$ vs. control).

RESULTS

EFFECTS OF HYPEROSMOTIC STRESS ON ASTROCYTES

Cell viability in the control group did not significantly change. Cell viability in the hyperosmotic group decreased with the hyperosmotic medium exposure time (Fig. 1).

CELL VOLUME IN RESPONSE TO HYPEROSMOTIC STRESS

Cell volume decreased in the hyperosmotic group compared with the control group, and cell volume is inveresly concentration dependent (Fig. 2A). Pre-treatment of cultures with U0126 or siRNA to down-regulation of ERK, or pre-treatment of cultures with SB203580 or siRNA to down-regulation of p38, the cell volume increased compared with only the hyperosmotic medium. However, after SP600125 administration to down-regulation of JNK, the cell volume did not have significantly changed (Fig. 2B).

INDUCTION OF AQPs IN CULTURED RAT ASTROCYTES SUBJECTED TO HYPEROSMOTIC STRESS

Astrocytes were incubated with isosmotic control medium (final osmotic pressure 282.5 mmol/L) or hyperosmotic medium (320, 333, and 345 mmol/L) at the indicated times (1, 3, 6, 12, and 24 h) before total RNA isolation for real-time RT-PCR or harvesting for immunoblot analysis to examine the effects of hyperosmotic stress on the expression of AQPs mRNA and proteins. All AQP mRNAs and proteins were remarkably higher in the hyperosmotic group compared with the control group, and increased with osmolality (data not shown). Although AQPs expression is concentration-dependent, astrocyte viability decreased in a time-dependent manner, as shown by the MTT test. Therefore, astrocytes were treated with hyperosmotic medium (320 mmol/L) for various periods to analyze the time course of the hyperosmotic induction on AQPs mRNA expression. The present study showed that AQP4 and AQP9 mRNAs as well as their proteins peaked at 12 h after treatment with hyperosmotic medium, whereas AQP3, -5, -8 mRNAs as well as their proteins peaked at 6 h. The AQPs expression was not significantly different in the control group at the different examined time points (Figs. 3 and 4).

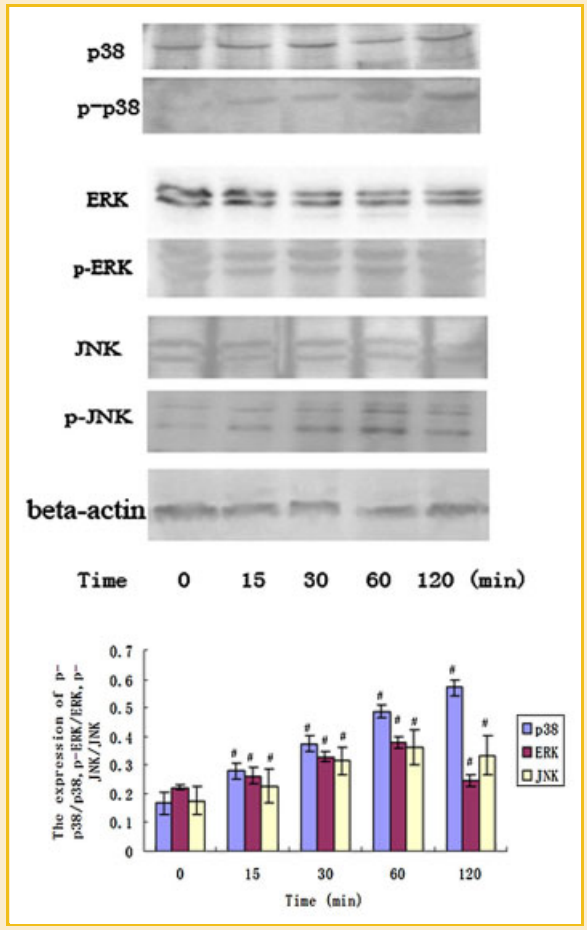

Fig. 5. Phosphorylation of MAPKs induced by hyperosmotic stress. Rat astrocytes were incubated in hyperosmotic medium (320 mmol/L). Cells were harvested and processed for immunoblotting using anti-p38 MAPK antibody or antiphosphorylated p38 MAPK (*p-p38*) antibody, anti-ERK antibody or anti-phosphorylated ERK (*p-ERK*) antibody, anti-JNK antibody or anti-phosphorylated JNK (*p-JNK*) antibody at the indicated times. Western blotting analysis of the ratio levels for the phosphorylation of MAPK to MAPK in astrocytes at 0, 15, 30, 60, and 120 min after exposure to hyperosmotic medium (#$P < 0.05$ vs. control).

ROLE OF MAPKs IN AQPs EXPRESSION IN CULTURED RAT ASTROCYTES SUBJECTED TO HYPEROSMOTIC STRESS

Hyperosmotic stress can activate signaling through three MAPK pathways. Immunoblot analyses of cells incubated in hyperosmotic medium were performed using antibodies that react with either the

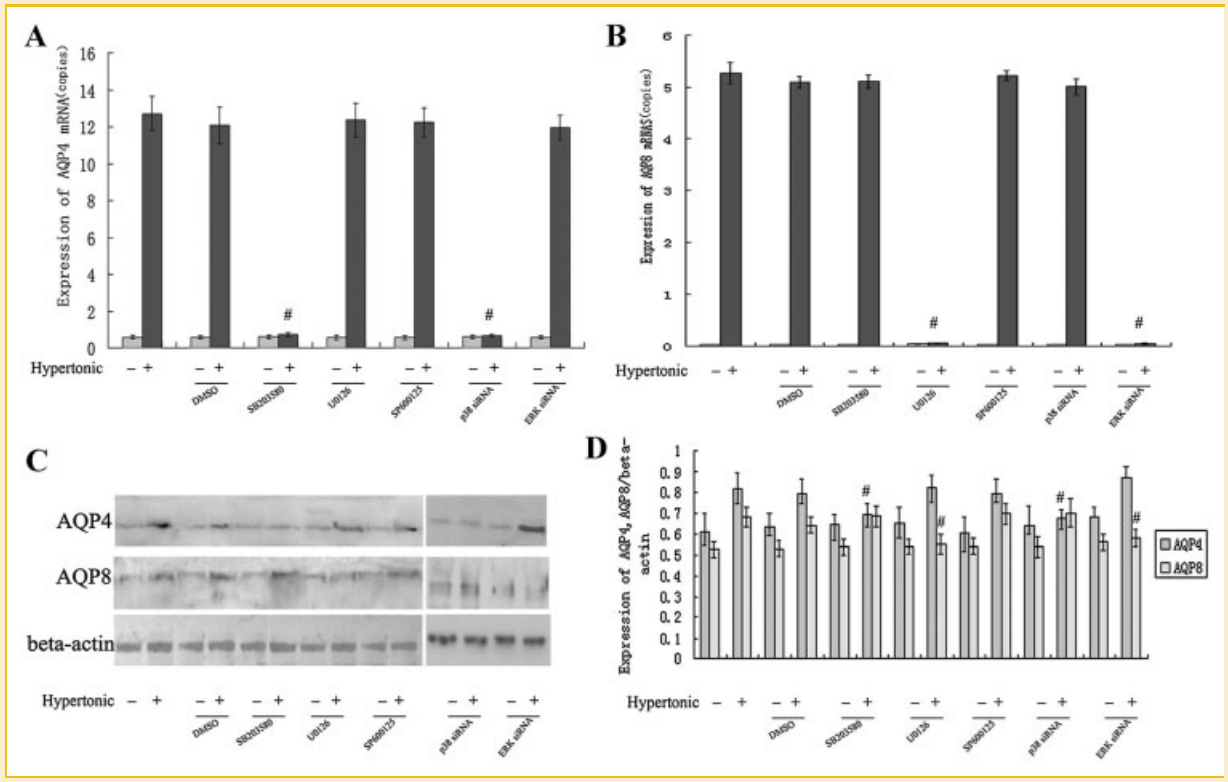

Fig. 6. Effect of MAPK inhibitor or siRNA on hyperosmotic induction of AQP3 and AQP4 mRNA and protein. Rat astrocytes were incubated for 6 h with or without hyperosmotic medium and MAPK inhibitors, p38 MAPK inhibitor SB203580 (10 μM) or p38 siRNA, ERK 1/2 inhibitor U0126 (10 μM) or ERK siRNA, or JNK inhibitor SP600125 (10 μM)). Astrocytes were also incubated with Me_2SO (*DMSO*) to reduce the possible effects of the DMSO vehicle. Cells were processed for real-time PCR of AQPs mRNA (A and B represent AQP3 and AQP4, respectively). The expression of mRNA (normalized to β-actin; $n = 5$ for each group). C: Rat astrocytes were treated as previously described and processed for immunoblotting with anti-AQP3 or anti-AQP4 antibody ($n = 5$ for each group; $\#P < 0.05$ vs. control).

phosphorylated or total amounts of each of the three MAPKs to investigate whether these three MAPKs are activated in rat astrocytes by hyperosmotic stress. The total amount of each MAPK remained constant, thus, p-ERK was activated by hyperosmotic stress after 15 min, and the activation persisted and reached its peak at 60 min. In addition, the p-p38 MAPK and p-JNK activation peaked at 120 and 60 min, respectively, after hyperosmotic exposure (Fig. 5). The possible involvement of the three MAPK cascades in the increased AQPs expression in response to hyperosmotic stress was investigated by examining the effects of the ERK inhibitor U0126 or siRNA, p38 MAPK inhibitor SB203580 or siRNA, and the JNK inhibitor SP600125. The hyperosmotic induction of AQP3, -5, and -8 mRNA and protein was inhibited by U0126 (10 μM) or ERK siRNA, whereas SB203580 (10 μM) or p38 siRNA inhibits the expression of AQP4 and AQP9. SP600125 (10 μM) had no effect on the expression of AQP3, -4, -5, -8, and -9 (Fig. 6). All three inhibitors had no effect on the basal expression of AQPs.

Astrocytes were incubated with the ERK activator, TPA (100 nm) under isosmotic conditions, to determine whether AQP3, -5, and -8 could be induced after ERK activation. ERK activation by TPA increased 15 min after exposure and peaked at 60 min, whereas TPA did not increase basal AQP3, -5, -8 expressions. These results suggest that ERK activation may be necessary and not sufficient for hyperosmotic induction of AQP3, -5, and -8 (Fig. 7).

Astrocytes were incubated with the p38 activator, anisomysin (50 μM) under isosmotic conditions, to determine that AQP4 and AQP9 could be induced after p38 activation. The total amount of p38 remained constant, and the activator caused its phosphorylation. p38 activation by anisomysin increased 30 min after exposure and then decrease quickly at 60 min. Anisomysin increased AQP4 and AQP9 mRNAs (Fig. 8).

DISCUSSION

To date, six aquaporin subtypes (AQP1, -3, -4, -5, -8, and -9) have been found in the rodent brain. Among them, AQP3, -4, -5, -8, and -9 are distributed in the mature astrocyte, which is the most distributed cell in the brain. However, most of the data came from studies on the expression of AQP4 and AQP9 [Frigeri et al., 1995ab; Badaut et al., 2002]. In the present study, the expression of AQP3, -5, -8 in hyperosmotic solutions are shown for the first time, with slightly different results from AQP4 and AQP9 at temporal variations after hyperosmotic solution treatment.

Hyperosmolatic hydration is a common clinical complication which is often caused by conditions, such as gravis brain injury, hyperosmolatic coma, or lasting hyperpyrexia. Hyperosmolatic hydration is mainly made up of intracellular hydration. A clinical symptom of this type of dehydration is that extracellular fluid does

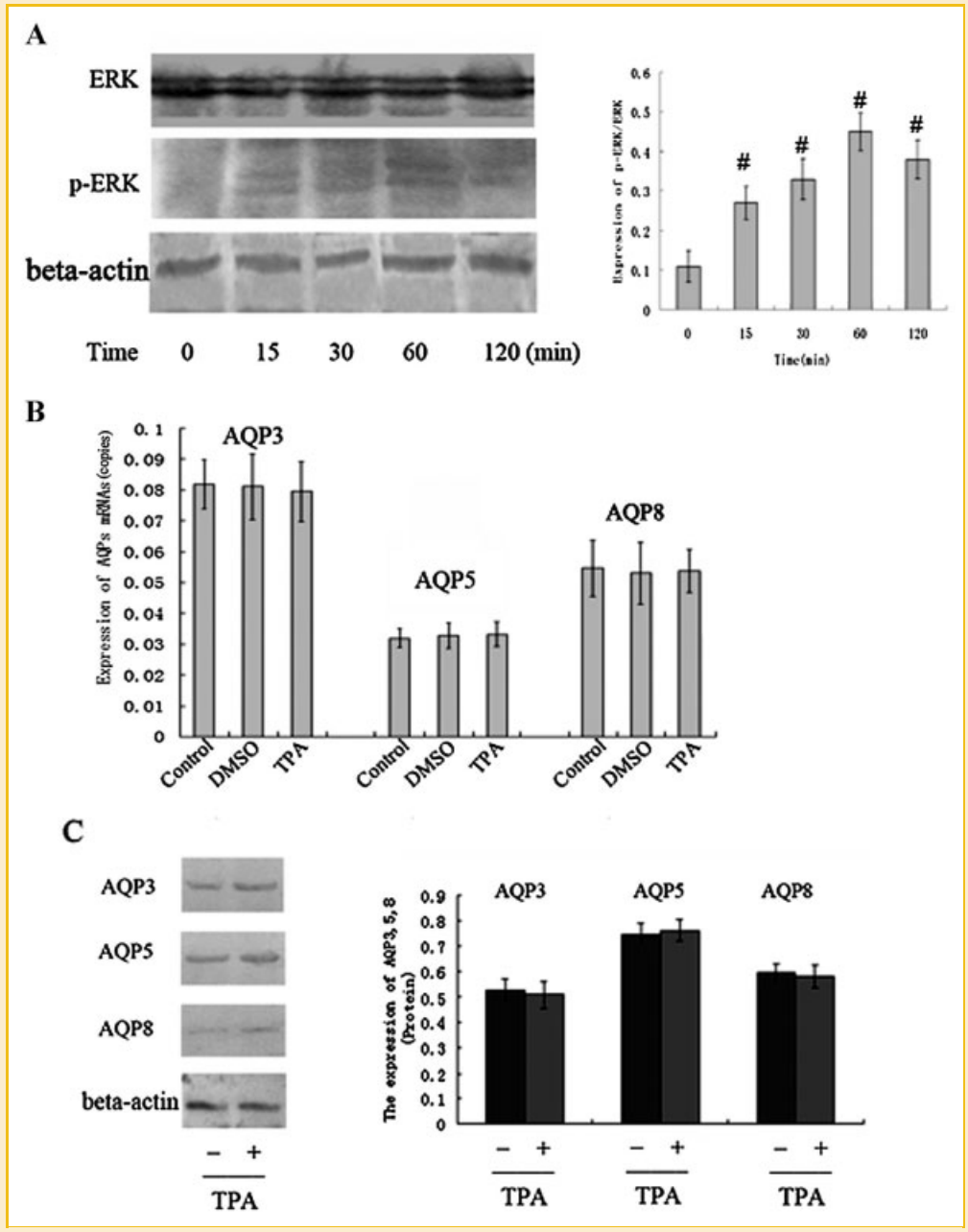

Fig. 7. Effect of ERK activators on hyperosmotic induction of AQP3, -5, and AQP8 mRNA and protein. A: Rat astrocytes were incubated in isosmotic medium containing TPA. Cells were harvested and processed for immunoblotting using anti-ERK antibody or anti-phosphorylated ERK (*p-ERK*). B: Rat astrocytes were incubated for 6 h with or without TPA under isosmotic conditions at the indicated times. Astrocytes were also incubated with Me_2SO (*DMSO*) to reduce the possible effects of the DMSO vehicle. Cells were processed for real-time PCR of AQP3, -5, or AQP8 mRNA. C: Rat astrocytes were incubated for 6 h with or without TPA under isosmotic conditions and processed for immunoblotting with anti-AQP3, -5, or anti-AQP8 antibody (#$P < 0.05$ vs. control).

not decrease severely. However, brain cell hydration can result in brain tissue shrinkage, dragging brain veins fracture, and consequently resulting in brain hemorrhage or subarachnoid hemorrhage. Therefore, the pathophysiological mechanism of hyperosmolatic hydration is an important topic for research, especially for water transport after hydration. This study demonstrates that hyperosmotic solutions to cultured astrocytes upregulates AQP mRNAs and proteins in a time-dependent manner with a peak of AQP4 and AQP9 at 12 h, and a peak of AQP3, -5, -8 at 6 h. Pre-treatment of cultures with U0126 or siRNA to down-regulation of ERK resulted in a marked inhibitor of AQP3, -5, -8 upregulation. Pre-treatment of cultures with SB203580 or siRNA to down-regulation of p38 reduced the increasing AQP4 and AQP9 expression.

Although five AQP subtypes were distributed in the same cell type, some AQPs reached their peaks differently after treatment with hyperosmotic medium, indicating that different AQPs possibly play different roles at different stages. In the early stage, all AQPs

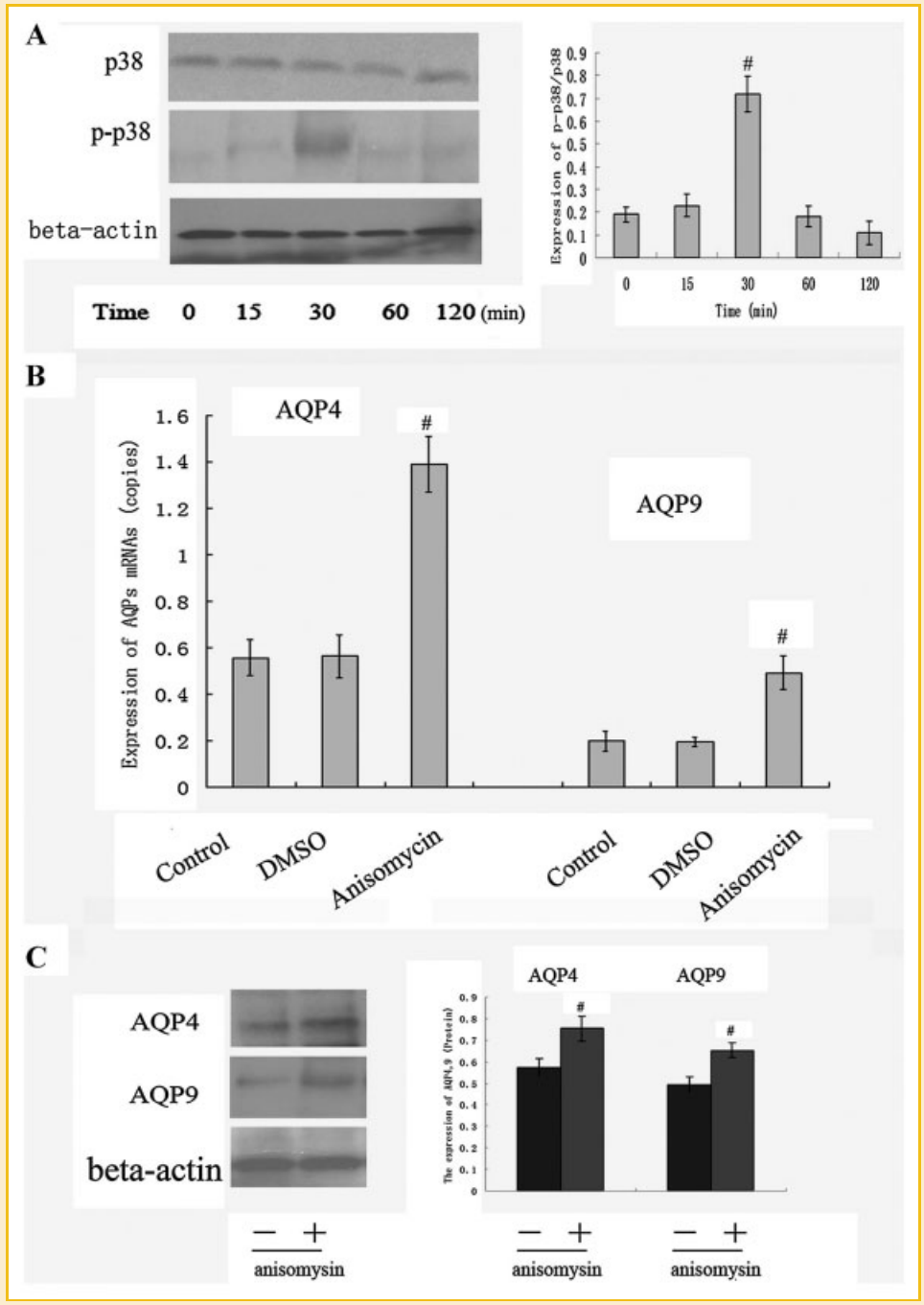

Fig. 8. Effect of p38 MAPK activators on the induction of AQP4 and AQP9 mRNAs and proteins. A: Rat astrocytes were incubated in isosmotic medium containing anisomysin. Cells were harvested and processed for immunoblotting using anti-p38 MAPK antibody or anti-phosphorylated p38 MAPK (*p-p38*) at the indicated times. B: Rat astrocytes were incubated for 6 h with or without anisomysin under isosmotic conditions. Cells were processed for real-time PCR of AQP4 or AQP9 mRNA. C: Rat astrocytes were incubated for 6 h with or without anisomysin under isosmotic conditions and processed for immunoblotting with anti-AQP4 or anti-AQP9 antibody (#$P < 0.05$ vs. >control).

increased quickly to adapt to the outside hyperosmotic stimuli. However, in the later stage, different AQPs showed different expression to hyperosmotic stimuli because of different regulated mechanisms and water-permeabilities.

The expression of AQPs reached their peaks, followed by a decrease after a certain period (6 or 12 h), indicating that AQPs expression reached their limitation at a certain time after hyperosmotic stimuli exposure. The limit could be compensatory for cell dehydration to relieve the hyperosmotic stimulation of cell structure and function injury within this time; however, at a longer period (6 or 12 h), the ability for AQPs to regulate the water may reach the maximum. Based on these results, the following conclusions can be drawn. First, AQPs expression was directly related to the extracellular hyperosmotic stimuli, suggesting that

AQPs are important in brain tissue water and electrolyte balance regulation. Second, the present study provided experimental basis for the new understanding of the pathophysiology of hyperosmotic dehydration mechanism, and guiding clinicians to grasp the correct time to treat rehydration. At present, the time for clinical treatment of hypernatremia is positioned at 48 h. At over 48 h, the adaptive brain response occurs, then is further reduced as plasma osmotic pressure causes water to enter the brain tissue, resulting in increased brain capacity, triggering cerebral edema, and a series of clinical symptoms. Our experiments showed that AQPs expression of astrocytes exposure to extracellular hyperosmotic solutions reach their peaks at 12 h, suggesting that enough water should be supplied in early rehydration to maintain normal cell structure and function.

MAPKs are important intracellular signal transduction pathways involved in the protective response of cells to hyperosmotic stress [Galcheva-Gargova et al., 1994; Han et al., 1994; Matsuda et al., 1995; Burg et al., 1996]. Under hyperosmotic conditions, AQP1 is regulated by all three MAPKs [Umenishi and Schrier, 2003], AQP4 and AQP9 are regulated by p38 [Arima et al., 2003], and AQP5 is regulated by ERK [Hoffert et al., 2000]. All three MAPKs were activated in our study, as indicated by Western blot using a phosphospecific antibody. In addition, the effects of p38 MAPK, ERK, and JNK inhibitors were examined for their effects on AQPs induction by hyperosmotic stress. Only the p38 inhibitor suppressed AQP4 and AQP9 expression, indicating that AQP4 and AQP9 expression in rat astrocytes under hyperosmotic conditions can be regulated by the p38 MAPK pathway, which is consistent with a previous study [Arima et al., 2003]. Meanwhile, anisomysin can increase AQP4 and AQP9 expression, which is different from the previous study [Arima et al., 2003]. The discrepancy may be related to the different hyperosmotic model. However, we could not exclude the possible function of anisomysin was performed though other signal pathway. The ERK inhibitor suppressed AQP3, -5, and -8 expression induced by hyperosmotic stress, however, the ERK activator TPA did not activate AQP3, -5, and -8 expression. Therefore, ERK activation maybe necessary, but not sufficient for AQP3, -5, and -8 expression. Likewise, we could not exclude that TPA performed its function through other signal pathway and its possible pleiotropic effects. Our results were slightly different from a previous study that reported AQP5 expression [Hoffert et al., 2000] The discrepancy can be related to the differences in the species (rat vs. mouse) or the cells (brain astrocyte vs. lung epithelial cells).

In conclusion, the present study supports the hypothesis that all AQPs in astrocytes are involved in hyperosmotic dehydration. With regard to the mechanisms by which AQPs mediate water transport in cultured astrocytes, the resulting unbalanced distribution of water and electrolytes inside and outside the cells possibly trigger the expression of MARK during exposure to the hyperosmotic cultured medium, which secondary-activated AQPs expression in astrocytes.

REFERENCES

Arima H, Yamamoto N, Sobue K, Umenishi F, Tada T, Katsuya H, Asai K. 2003. Hyperosmolar mannitol simulates expression of aquaporins 4 and 9 through a p38 mitogen-activated protein kinase-dependent pathway in rat astrocytes. J Biol Chem 278:44525–44534.

Badaut J. Aquaglyceroporin 9 in brain pathologies. Neuroscience 168:1047–1057.

Badaut J, Lasbennes F, Magistretti PJ, Regli L. 2002. Aquaporins in brain: Distribution, physiology, and pathophysiology. J Cereb Blood Flow Metab 22:367–378.

Burg MB, Kwon ED, Kultz D. 1996. Osmotic regulation of gene expression. FASEB J 10:1598–1606.

Dmitrieva NI, Burg MB. 2005. Hypertonic stress response. Mutat Res 569: 65–74.

Frigeri A, Gropper MA, Turck CW, Verkman AS. 1995a. Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. Proc Natl Acad Sci USA 92:4328–4331.

Frigeri A, Gropper MA, Umenishi F, Kawashima M, Brown D, Verkman AS. 1995b. Localization of MIWC and GLIP water channel homologs in neuromuscular, epithelial and glandular tissues. J Cell Sci 108(Pt 9):2993–3002.

Fu X, Li Q, Feng Z, Mu D. 2007. The roles of aquaporin-4 in brain edema following neonatal hypoxia ischemia and reoxygenation in a cultured rat astrocyte model. Glia 55:935–941.

Galcheva-Gargova Z, Derijard B, Wu IH, Davis RJ. 1994. An osmosensing signal transduction pathway in mammalian cells. Science 265:806–808.

Han J, Lee JD, Bibbs L, Ulevitch RJ. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808–811.

Hoffert JD, Leitch V, Agre P, King LS. 2000. Hypertonic induction of aquaporin-5 expression through an ERK-dependent pathway. J Biol Chem 275:9070–9077.

Kato T, Chiu TC, Lim R, Troy SS, Turriff DE. 1979. Multiple molecular forms of glia maturation factor. Biochim Biophys Acta 579:216–227.

Li YH, Sun SQ. 2004. [Influence of hypertonic medium on aquaporin 4 mRNA expression in astrocytes in vitro]. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 16:210–213.

Maric K, Wiesner B, Lorenz D, Klussmann E, Betz T, Rosenthal W. 2001. Cell volume kinetics of adherent epithelial cells measured by laser scanning reflection microscopy: Determination of water permeability changes of renal principal cells. Biophys J 80:1783–1790.

Matsuda S, Kawasaki H, Moriguchi T, Gotoh Y, Nishida E. 1995. Activation of protein kinase cascades by osmotic shock. J Biol Chem 270:12781–12786.

Papadopoulos MC, Krishna S, Verkman AS. 2002. Aquaporin water channels and brain edema. Mt Sinai J Med 69:242–248.

Schmidt E, Schimmel P. 1994. Mutational isolation of a sieve for editing in a transfer RNA synthetase. Science 264:265–267.

Umenishi F, Schrier RW. 2003. Hypertonicity-induced aquaporin-1 (AQP1) expression is mediated by the activation of MAPK pathways and hypertonicity-responsive element in the AQP1 gene. J Biol Chem 278:15765–15770.

Venero JL, Vizuete ML, Machado A, Cano J. 2001. Aquaporins in the central nervous system. Prog Neurobiol 63:321–336.

Verkman AS. Aquaporins in clinical medicine. Annu Rev Med 63:303–316.

Yamamoto N, Yoneda K, Asai K, Sobue K, Tada T, Fujita Y, Katsuya H, Fujita M, Aihara N, Mase M, Yamada K, Miura Y, Kato T. 2001. Alterations in the expression of the AQP family in cultured rat astrocytes during hypoxia and reoxygenation. Brain Res Mol Brain Res 90:26–38.

Yang M, Gao F, Liu H, Yu WH, Sun SQ. 2009. Temporal changes in expression of aquaporin-3, -4, -5 and -8 in rat brains after permanent focal cerebral ischemia. Brain Res 1290:121–132.

Yoneda K, Yamamoto N, Asai K, Sobue K, Fujita Y, Fujita M, Mase M, Yamada K, Nakanishi M, Tada T, Miura Y, Kato T. 2001. Regulation of aquaporin-4 expression in astrocytes. Brain Res Mol Brain Res 89:94–102.