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Selective Induction of Glial Glutamate Transporter GLT-1 by Hypertonic Stress in C6 Glioma Cells

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Glial glutamate transporter GLT-1 mRNA was selectively induced in C6 glioma cells exposed to hypertonic stress (HS), while the expression of two other subtypes, GLAST and EAAC1, was suppressed. HS increased phosphorylation of the MAPK family, ERK, p38 MAPK, and JNK. Treatment with a PKC inhibitor showed that phosphorylation of both p38 MAPK and JNK is PKC-dependent but ERK phosphorylation is independent. Inhibition of either ERK or p38 MAPK did not abolish GLT-1 mRNA induction. Inhibition of PKC also had no effect. These findings indicate that the induction of GLT-1 mRNA by HS is independent of the MAPK pathways. This is the first report that the expression of glial glutamate transporters is osmotically regulated.

In the mammalian central nervous system (CNS), glutamate is the main neurotransmitter used by most excitatory synapses and the removal of glutamate from the synaptic cleft is essential both for termination of the synaptic action and for protection of neurons from excitotoxicity (1). Concentrative uptake of extracellular glutamate is mediated by high affinity Na^+-dependent transporters located in the plasma membrane of neuronal and glial cells (2). Three Na^+-dependent transporter subtypes have been cloned; GLAST (EAAT1), GLT-1 (EAAT2), and EAAC1 (EAAT3) (3–5). GLAST and GLT-1 are predominantly localized in glial cells (5, 6), whereas EAAC1 is selectively expressed in neurons (3). EAAT4, a fourth subtype, has also been identified in human cerebellar neurons (7).

The failure or loss of these transporters may be involved in various neurological diseases. For instance, brain ischemia and traumatic injury cause the loss of the GLT-1 and GLAST proteins (8, 9). A selective reduction in the GLT-1 protein was observed in the motor cortex and spinal cord of patients with amyotrophic lateral sclerosis (ALS) (10). Also, amyloid β peptide inhibited glutamate uptake (11).

Various stress stimuli, such as oxidative stress and ischemia, have been shown to inhibit glutamate uptake, mediated by the failure of ATP-driven transport or by post-translational modification of the transporter (12–14). The effect of such stress stimuli on the expression of glutamate transporters, however, is still unclear. From the pathogenic and therapeutic aspects, clarification of the regulatory mechanism of expression is an important issue. In the present study, we have investigated the expression of glutamate transporters in C6 glioma cells exposed to stress stimuli and have found the selective induction of GLT-1 mRNA by hypertonic stress (HS).

MATERIALS AND METHODS

Reagents. Anti-extracellular signal-regulated kinase (ERK) 1/2, -phospho-specific ERK1/2 (Tyr-204), -c-Jun N-terminal kinase (JNK), and -phospho-specific JNK (Thr-183/Tyr-185) antibodies were purchased from New England Biolabs (Beverly, MA). Anti-p38 mitogen-activated protein kinase (MAPK) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-specific p38 MAPK (Thr-180/Tyr-182) antibody was from Promega (Madison, WI). PD098059, a MEK1 inhibitor, was also obtained from New England Biolabs. SB 203580, a p38 MAPK inhibitor, was from Calbiochem (La Jolla, CA), and chelerythrine chloride, a protein kinase C (PKC) inhibitor, was from RBI chemicals (Natick, MA).

Cell culture. C6 glioma cells (ATCC CCL107) were maintained at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. When cells reached 80% confluency after 3 days culture, they were exposed to various stress stimuli; hypertonic stress (addition of 150 mM NaCl or 200 mM sucrose to normal culture medium), chemical ischemia (1 mM sodium azide and 1 mM 2-deoxyglucose in glucose-free medium for 2 h), excess potassium (substitution of 100 mM KCl for NaCl), and oxidative stress (1 mM hydrogen peroxide for 1 h). Excess potassium has shown to induce glial swelling, mimicking ischemic conditions (15).

RNA preparation and quantitative RT-PCR. Total cell RNA was extracted using the ISOGEN RNA Isolation Kit (Nippon Gene, Japan) originally described by Chomczynski (16). The concentration of the isolated RNA was determined spectrophotometrically at 260 nm. cDNA was synthesized in a 20 μl reaction mixture containing 500 ng of total RNA, 1 mM dNTPs, 5 mM $MgCl_2$, 20 units of RNAase inhibitor, 5 units of Avian Myeloblastosis Virus (AMV) reverse transcriptase, and 0.125 μM of Oligo d(T). Reverse transcription (RT)

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was carried out by incubation at 42°C for 30 min followed by denaturation at 99°C for 5 min and quick-chilled on ice. PCR was performed in a final volume of 25 μl containing 5 μl RT product, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 units/100 μl Taq DNA polymerase, and 0.2 μM each of sense and antisense primers as follows: EAAC1 sense 3′-TGTCACCACGGCCATGACAA-5′, antisense 3′-CGTTCACCATGGTCCTGAAC-5′ (nt +549 to +1351); GLT-1 sense 3′-GACAGCCACCTCAGCTCCGA-5′, antisense 3′-ACTCCACCATCAGCTTGGCC-5′ (nt +58 to +862); GLAST sense 3′-CTACTCACCGTCAGCGCTGT-5′, antisense 3′-AGCACAAATCTGGTGATGCG-5′ (nt +154 to +1172). The amplification protocol was as follows; initial denaturation at 94°C for 3 min; 10–35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; a final extension at 72°C for 7 min using the GeneAmp 2400 thermal cycler (Perkin Elmer, Applied Biosystem Inc., Forster City, CA). 10 μl of PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide (0.5 μg/ml). Then, the PCR products were visualized using a UV transilluminator coupled to a CCD camera and analyzed by quantitative densitometry using a computerized image analysis program (NIH IMAGE 1.51).

Western blotting. The cells were washed with ice-cold phosphate-buffered saline twice, scraped, and lysed with buffer [50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% (wt/vol) 2-mercaptoethanol, 1% Triton-X, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropylfluorophosphate, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 mM benzamidine, 0.1 mM orthovanadate, and 1 mM $(NH_4)_6Mo_7O_{24}$, pH 7.5]. The lysates were centrifuged at 20,000g for 30 min and the supernatants were collected for analysis. Samples containing equal amounts of protein (20 μg) were electrophoresed on polyacrylamide gels (8–16%) in the presence of SDS. Immunoblotting was carried out by transferring the proteins to polyvinylidene difluoride microporous membrane, blocking with 5% nonfat dry milk in 50 mM Tris-buffered saline containing 0.1% Tween-20 (TBS-T), and incubating overnight at 4°C with the primary antibodies. The blots were then washed in TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, UK) in TBS-T for 1 h at room temperature. The specific reaction was visualized using the enhanced chemiluminescence (ECL) method (Amersham).

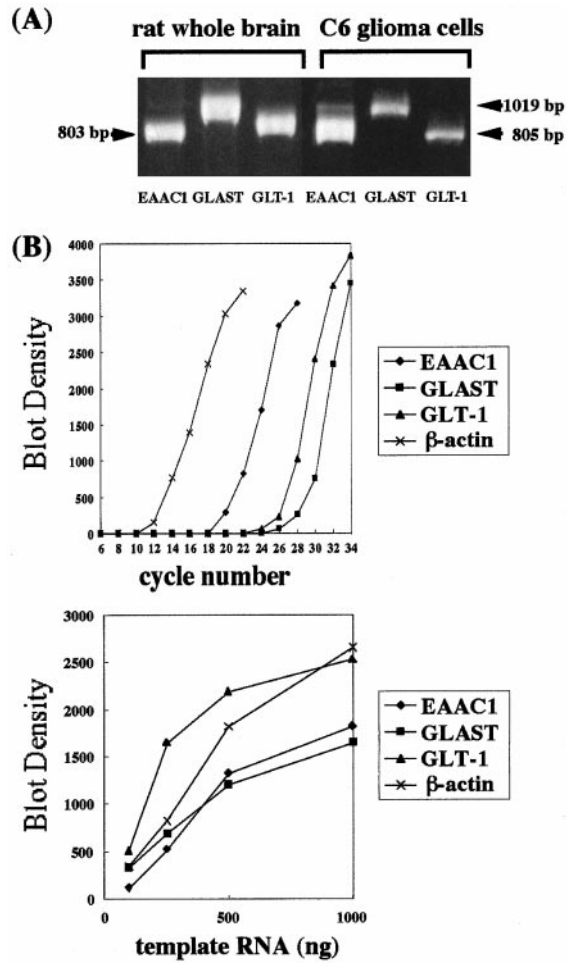

FIG. 1. (A) Expression of EAAC1, GLAST, and GLT-1 mRNA in C6 glioma cells and rat brain. 803 bp, 1019 bp, and 805 bp RT-PCR products amplified from 500 ng of total RNA (30 cycles) corresponded to EAAC1, GLAST, and GLT-1. (B) The relationship between the optical density units of the PCR products and either PCR cycle number (upper panel) or input RNA concentration (lower panel). β-actin mRNA was also analyzed as an internal standard.

RESULTS

Effect of various stress stimuli on the expression of glutamate transporter mRNA in C6 glioma cells. RT-PCR analysis demonstrated that C6 glioma cells expressed all three subtypes of glutamate transporter, EAAC1, GLT-1, and GLAST. Each PCR product was sequenced to confirm that glutamate transporters were adequately amplified (data not shown). A comparison of the PCR products of three glutamate transporters amplified from rat whole brain RNA showed that EAAC1 was the predominant subtype of glutamate transporter in C6 cells (Fig. 1A). For semiquantitative RT-PCR analysis, we first examined the relationship between the PCR cycle number and the optical density units of the PCR products. The optimal PCR cycle number within a linear range of amplification was determined as follows: 14 cycles for β-actin as an internal standard, 24 cycles for EAAC1, 30 cycles for GLT-1, and 32 cycles for GLAST. We next made a quantitative analysis of input RNA at various concentrations. The rate of amplification was almost exponential from 100 to 1000 ng of input RNA for each primer set at the determined cycle number. Therefore, all subsequent studies were performed using 500 ng of input RNA (Fig. 1B).

Semi-quantitative RT-PCR analysis demonstrated that exposure to chemical ischemia, oxidative stress, or excess potassium decreased the expression of all three glutamate transporters. On the other hand, addition of 200 mM sucrose to the culture medium increased the expression of GLT-1 mRNA, while EAAC1 and GLAST mRNA expression was suppressed. 150 mM NaCl had the same effect on the expression of the glutamate transporters as 200 mM sucrose (Fig. 2), suggesting that hypertonicity induces GLT-1 mRNA.

Time course of GLT-1 mRNA induction and an increase in the phosphorylation of MAP kinase family by hypertonic stress. Next we studied the expression of GLT-1 mRNA exposed to HS at various time points. The induction of GLT-1 mRNA was found at 12 h after

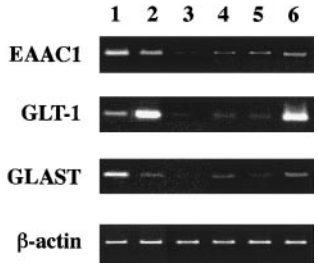

FIG. 2. Expression of EAAC1, GLT-1, and GLAST mRNA in C6 glioma cells exposed to various stress stimuli. Lane 1, control. Lane 2, HS (200 mM sucrose). Lane 3, excess potassium (substitution of 100 mM KCl for NaCl). Lane 4, oxidative stress (1 mM hydrogen peroxide for 1 h). Lane 5, chemical ischemia (1 mM sodium azide and 1 mM 2-deoxyglucose in glucose-free medium for 2 h). Lane 6, HS (150 mM NaCl). Cells were harvested 24 h after exposure and the expression of glutamate transporters was analyzed by semiquantitative RT-PCR analysis. Note the selective induction of GLT-1 mRNA by HS. The results shown are representative of three independent experiments.

exposure and continued until 24 h (Fig. 3A). The MAPK family has been shown to play an essential role in the osmosignaling pathway. Activation of MAPK requires phosphorylation. Immunoblot analysis revealed that the phosphorylated isoform of ERK, p38 MAPK, and JNK increased in C6 glioma cells exposed to HS, indicating that they were activated. The phosphorylation of ERK, JNK, and p38 MAPK were peaked at 30 min–1 h after exposure and sharply decreased until 12 h (Fig. 3B).

Induction of GLT-1 mRNA was independent of protein kinase C and MAPK family. To investigate the involvement of the MAPK family in HS-mediated induction of GLT-1 mRNA, C6 glioma cells exposed to HS were co-incubated with either PD098059, a MEK1 inhibitor, or SB203580, a p38 MAPK inhibitor. The presence of 50 μM PD098059 almost completely abolished the phosphorylated form of ERK. PD098059 slightly decreased phosphorylated p38 MAPK but had no effect on JNK phosphorylation. Application of 100 μM SB203580 decreased the HS-induced phosphorylation of p38 MAPK although the detectable level of phosphorylated p38 still be remained. SB203580 had no effect on ERK phosphorylation but slightly increased JNK phosphorylation. To clarify whether PKC is involved in HS-mediated MAPK pathways and/or GLT-1 mRNA induction, the effect of chelerythrine, a selective PKC inhibitor, was also studied. Inhibition of PKC by chelerythrine resulted in a decrease in both p38 MAPK and JNK phosphorylation in a concentration-dependent manner. In contrast, the phosphorylated form of ERK was increased in the presence of chelerythrine (Fig. 4).

Semiquantitative RT-PCR analysis demonstrated that HS increased GLT-1 mRNA by approximately 7-fold but suppressed both EAAC1 and GLAST mRNA about by 50% compared with controls. 50 μM PD098059 did not attenuate the HS-mediated GLT-1 mRNA induction. The presence of 100 μM SB203580 partially blocked the GLT-1 mRNA induction, but 100 μM SB203580 also decreased β-actin mRNA, an internal standard, and the change in the ratio GLT-1/β-actin was not significant. We also tested the treatment with 1 μM and 10 μM SB203580 and found no effect on the GLT-1 mRNA induction (data not shown). The suppression of both EAAC1 and GLAST mRNA by HS was also unchanged by either PD098059 or SB203580. Furthermore, 10 μM chelerythrine, which almost completely inhibited the phosphorylation of both p38 MAPK and JNK, had no effect on expression of the glutamate transporters (Fig. 5).

DISCUSSION

Some previous studies have demonstrated that C6 glioma cells express EAAC1, a neuronal glutamate transporter, but not GLT-1 or GLAST (17, 18). Conversely, Casado *et al.* have reported that C6 cells ex-

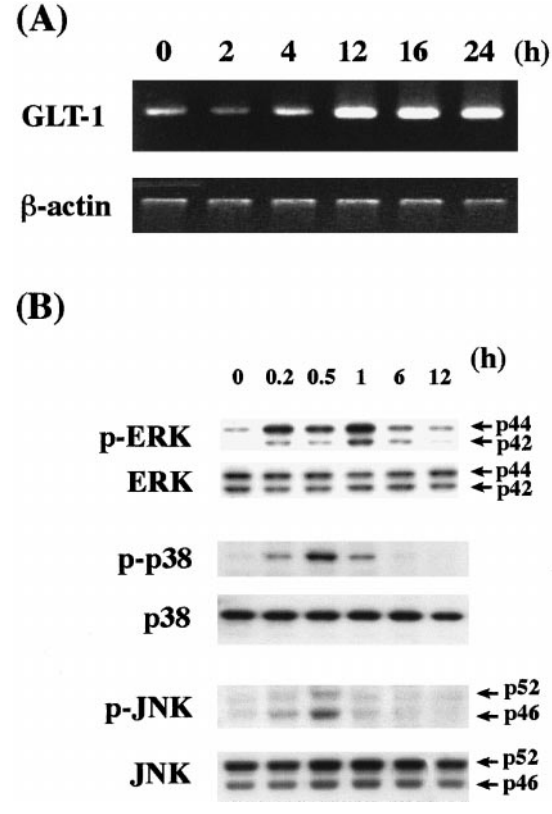

FIG. 3. (A) Time course of GLT-1 mRNA induction by HS. (B) An increase in phosphorylation of the MAP kinase family; ERK, p38 MAPK, and JNK by HS. Equal amounts of proteins (20 μg) extracted from cells were analyzed by immunoblot using either phospho-specific (*P*-) or antibodies recognizing both the phosphorylated and nonphosphorylated forms of ERK, p38 MAPK, and JNK to detect the amount of phosphorylated or total protein. The results shown are representative of three independent experiments.

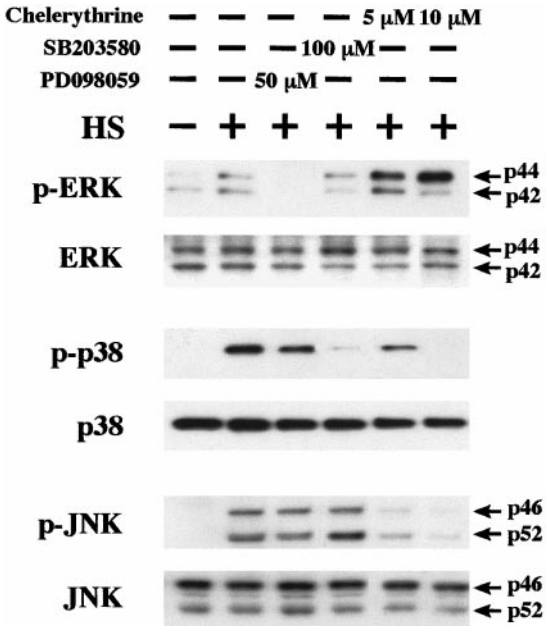

FIG. 4. Effect of various inhibitors on the phosphorylation of the MAPK family. After a 1-h preincubation with inhibitors, cells were exposed to HS for 30 min in the presence or absence of inhibitors and analyzed by immunoblot. The results shown are representative of three independent experiments.

press GLT-1 (19). Our RT-PCR analysis showed that C6 glioma cells express all three subtypes of glutamate transporter mRNA and that EAAC1 is the predominant isoform. The differential expression of glutamate transporters in C6 glioma cells may be explained by the finding that C6 glioma cells have a variable mixed phenotype which depends on factors such as the number of passages, lines, or culture conditions (20, 21). After exposure to a variety of stress stimuli, including chemical ischemia, high potassium stimulation, and oxidative stress, the expression of all three glutamate transporters was suppressed. Interestingly, short-term exposure to chemical ischemia (2 h) or oxidative stress (1 h) resulted in a prolonged suppression. The reduced expression of glutamate transporters has been reported *in vivo* in brain injury, such as ischemia and trauma (8, 9), which may be involved in delayed neuronal damage. It remains undetermined whether the prolonged reduction of glutamate transporter mRNA after exposure to short-term stress stimuli is due to transcriptional inhibition or an increased turnover rate.

Unlike other stress stimuli, HS selectively induced GLT-1 mRNA. Previous studies have revealed that a change in extracellular osmolarity results in the altered expression of several genes in mammalian cells (22). In particular, it has been noted that organic osmolyte transporters, such as a Na-myo-inositol cotransporter, a betaine transporter, and a taurine transporter, are dramatically induced in several types of cells, including glial cells (23–25). Accumulation of organic osmolytes is beneficial for cells to maintain their volume. Glutamate can act as an organic osmolyte (26), and an increase in the uptake of glutamate has been reported in renal cells exposed to HS (27). Therefore, it is possible that the induction of GLT-1 mRNA in C6 glioma cells is one of several responses to HS for the accumulation of organic osmolytes. In contrast to the induction of GLT-1 mRNA, the expression of GLAST mRNA and EAAC1 mRNA were suppressed. Although the mechanism of the differential regulation of each glutamate transporter subtype is unknown, it is likely that the selective induction of GLT-1 mRNA by HS is cell-type specific because renal cells have shown to increase EAAC1 mRNA in response to HS (27). Several organic osmolyte transporters have an osmotic response element in the 5′ flanking region, which is essential for an increase in the transcription in re-

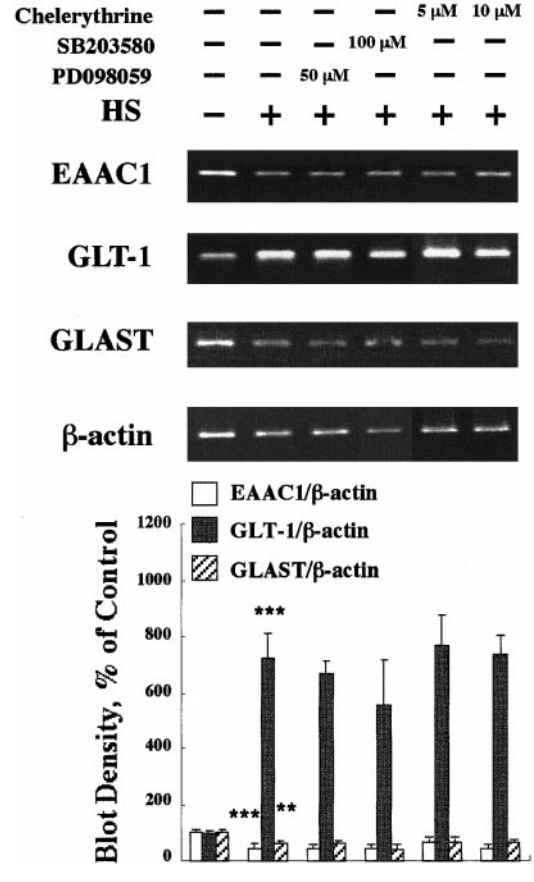

FIG. 5. Effect of various inhibitors on GLT-1 mRNA induction by HS. After a 1-h preincubation with inhibitors, cells were exposed to HS for 12 h in the presence or absence of inhibitors and analyzed by semiquantitative RT-PCR. The upper panel shows the typical blots of glutamate transporter mRNA and the lower panel shows the results of the densitometric analysis. The optical densitiy of each blot was normalized to that of β-actin mRNA. Data represent mean ± SEM (n = 6–9). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control, using Student's *t* test.

sponse to HS (28, 29). To determine whether such element exists in the GLT-1 gene awaits future study.

The MAPK family is thought to play an important role in the signaling pathway for the osmoregulation of gene expression. For example, inhibition of p38 MAPK abolished the induction of mRNAs for HSP70 and a betaine transporter by HS (30). In the present study, ERK, p38 MAPK, and JNK were all transiently activated in C6 glioma cells exposed to HS. Inhibition of PKC by chelerythrine resulted in a decrease in the HS-induced phosphorylation of JNK and p38 MAPK but ERK phosphorylation was slightly increased. This indicated that activation of JNK and p38 MAPK by HS is PKC-dependent but ERK activation is PKC-independent in C6 glioma cells. Activation of the MAPK family by osmotic stimulation has been studied in several types of cells, but its PKC dependency is not uniform and may depend on the cell-type (31–36). It has been reported previously that HS-mediated clustering and internalization of cell surface receptors is responsible for JNK activation (37). It is possible that the cell-type dependent osmosignaling cascade results from the differential expression of a variety of receptors.

Although HS activated MAPK signaling pathways in C6 glioma cells, inhibition of either ERK or p38 MAPK did not significantly attenuate the induction of GLT-1 mRNA. Although SB203580, a p38 MAPK inhibitor, slightly decreased GLT-1 mRNA, the change was not significant after normalization with internal standard. Also, SB203580 did not reverse the decrease of both EAAC1 and GLAST mRNA by HS. Furthermore, inhibition of PKC, resulting in a decrease in both JNK and p38 MAPK phosphorylation, also had no effect. Although it remains possible that these inhibitors may affect other signaling pathway nonspecifically, the activation of ERK, p38 MAPK, and JNK were effectively suppressed as shown by Western blot. We concluded, therefore, that the induction of GLT-1 mRNA by HS is independent of these pathways. The induction of organic osmolyte transporters, such as a Na-myo-inositol cotransporter and a betaine transporter, by HS is p38 MAPK-dependent but ERK-independent (30, 36), whereas the induction of aldose reductase by HS is independent of both p38 MAPK and JNK (38). HS is also known to activate other signaling pathways, including protein kinase A (39), the Janus kinase/signal transducer and activator of transcription (JAK/STAT) (40), and cyclic GMP (41). In particular, the level of intracellular cyclic AMP (cAMP) has been shown recently to regulate GLT-1 mRNA in cultured astrocytes (42). Treatment with dibutyryl cAMP, a cell-permeable cAMP analog, had little effect on GLT-1 mRNA in C6 glioma cells (data not shown), but these pathways are thought to have complex cross-talk with each other. Further investigation will be needed to clarify whether such pathways are involved in the induction of GLT-1 mRNA.

This is the first report, to our knowledge, that the expression of glutamate transporters in glial cells is regulated by osmotic stimulation. Whether glutamate transporters *in vivo* are also osmotically regulated remains an unsettled issue. Our current findings, however, indicate the possibility that some pathological conditions disrupting the regulation of extracellular osmolarity such as metabolic encephalopathy and ischemic injury can affect the expression of glutamate transporters, modulating the level of extracellular glutamate and neuronal survival.

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