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Endothelin Downregulates the Glutamate Transporter GLAST in cAMP-Differentiated Astrocytes In Vitro

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ABSTRACT Endothelin (ET) is a putative pathogenetic mediator associated with brain trauma and ischemia. Because a link between neuronal damage after these injuries and glial Na^+-dependent L-glutamate transporter activity has been suggested, we investigated the effect of ET on the glutamate clearance ability of astrocytes. Dibutyryl cyclic adenosine monophosphate (dBcAMP), which is widely used to induce differentiation of cultured astrocytes, markedly increased [^{3}H]glutamate transport activity in a concentration- and time-dependent manner. In the presence of ET, however, dBcAMP decreased the glutamate uptake. This effect was efficiently prevented by an antagonist of ET_B receptor, but not of ET_A receptor. ET per se was virtually ineffective. Eadie–Hofstee analysis demonstrated that dBcAMP increased the V_{max} value of glutamate uptake activity by 43.4% in the absence of ET, but decreased it by 41.4% in the presence of ET, without apparent changes in the K_m value. Accordingly, Western blot analysis indicated that the change in transport activity correlated closely with that in expression level of the glial glutamate transporter GLAST. These results may represent the mechanisms by which ET aggravates trauma- and ischemia-elicited neuronal damage. *GLIA 37:178–182, 2002.*

Endothelin (ET) was originally identified as a potent vasoconstrictor peptide produced by vascular endothelial cells (Rubanyi and Polokoff, 1994) and has been implicated in brain trauma and ischemia. In the central nervous system, ET and its receptor are located predominantly in astrocytes (Hori et al., 1992). Recent evidence has shown that expression levels of ET and the receptor are enhanced after various types of brain injury (Willette et al., 1993; Bian et al., 1994; Barone et al., 1994; Uesugi et al., 1996; Pluta et al., 1997), and also that exogenous ET application exacerbates ischemic brain damage (Agnati et al., 1991; Gartshore et al., 1997). In addition, pharmacological blockade of ET receptor efficiently attenuates ischemic damage (Barone et al., 1995; Tatlisumak et al., 1998; Pfister et al., 2000), suggesting that endogenous ET serves as a pathogenetic mediator in brain trauma and ischemia.

Extracellular glutamate is rapidly removed by astrocytes by the high-affinity Na^+-dependent L-glutamate transporters GLAST and GLT-1. Thus, this ability of astrocytes is critical in preventing excessive accumulation of glutamate and protecting neurons against excitotoxicity (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998). Indeed, brain damage in traumatic and ischemic injuries is well associated with a significant decrease in the levels of astrocytic glutamate transporters (Rao et al., 1998; Yin et al., 1998). In the present study, we addressed the possible contribu-

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tion of ET to glutamate transport activity of cultured astrocytes.

Agents used in the present study included endothelin-1 (Peptide Institute, Osaka, Japan), dibutyryl cyclic AMP (dBcAMP) (Sigma, St. Louis, MO), BQ123 (RBI, Natick, MA), BQ788 (RBI), DL-threo-β-hydroxy aspartate (Sigma), rhodamine phalloidin (20 U/ml) (Molecular Probes, Eugene, OR), peroxidase-conjugated anti-rabbit IgG antibody (Sigma), mouse anti-glial fibrillary acidic protein (GFAP) antibody (Amersham, Buckinghamshire, UK), and L-[^{3}H]glutamate (Amersham).

Cortical astrocytes were prepared from postnatal 2-day-old rat pups. Cortical hemispheres were dissected out and pooled into Leibovitz's L-15 medium. After removing meninges, they were dissociated by 0.25% trypsinization. The cell suspension was then centrifuged at 250*g* for 5 min, and the pellet was resuspended in Eagle's minimum essential medium with 10% fetal bovine serum (FBS) and placed into 75-cm^2 culture flasks at a density of 1×10^6 cells/cm^2. Cultures were maintained in a humidified, 5% CO_2 incubator at 37°C. The medium was changed every 3–4 days. After the cultures became confluent, cells were detached from the flask with trypsin/ethylenediaminetetraacetic acid solution (Life Technologies, Grand Island, NY), and plated onto 24-well culture plates at a density of 2×10^4 cells/cm^2. At 12 days in vitro, the cultures were treated with a low concentration (3%) of FBS for 24 h. Drugs were administered and were thereafter replaced with fresh drugs every 3 days. On this schedule of drug replacement, the ET effect is not affected by the stability of ET, as we confirmed that the effect of fresh ET could be reproducible by the old ET that underwent cell-free incubation at 37°C for 4.5 days (data not shown).

To observe astrocytic morphology, the cells were fixed with 4% paraformaldehyde, incubated overnight with anti-GFAP antibody (1:1,000), and stained with a Vectastain ABC kit (Vector; Burlingame, CA). For F-actin staining, the fixed cells were treated with rhodamine phalloidin (20 U/ml) at room temperature for 30 min. Fluorescence images were obtained with a laser scanning confocal system Micro Radiance (Bio-Rad, Hercules, CA).

L-[^{3}H]glutamate uptake was measured as described elsewhere (Swanson et al., 1997). After cultures were washed for 30 min with modified Hank's balanced salt solution (HBSS) composed of 137 mM NaCl, 0.34 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, and 2 mM glucose at pH 7.4, and then exposed to [^{3}H]glutamate (0.25 μCi/ml) and 10 μM-unlabeled glutamate for 7 min, uptake was terminated by ice-cold HBSS. Cultured cells were lysed in 0.5 N NaOH. Aliquots were taken for scintillation counting and for protein assays (Lowry et al., 1951).

For Western blot analysis, cells were washed twice with cold phosphate buffered saline (PBS) and then harvested. After intense sonication (23 kHz, 50 W, 1 min ×3) with the cell disruptor MS50 (Heat systems-Ultrasonics, NY), cell suspensions were centrifuged at 800*g* for 5 min at 4°C. An aliquot of this supernatant was removed for protein assay. Another aliquot was diluted in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein (1 μg) were loaded onto each lane. Protein samples and molecular mass markers were separated by electrophoresis on 10% polyacrylamide-SDS gels and transferred onto polyvinylidene difluoride membrane. The membranes were incubated in PBS containing 0.5% Tween 20 and 5% skim milk at room temperature for 1 hr and then with anti-GLAST antibody (1:1,000) at 4°C overnight. The membranes were washed for 30 min and incubated with peroxidase-conjugated anti-rabbit IgG antibody (1:5,000) for 1 h at room temperature. Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL) kit (NEN, Boston, MA). The density of immunoreactive bands was quantified by an NIH Image program. Anti-GLAST antibody recognized a protein with a molecular weight of ~64 kDa. Few immunoreactivities of multimeric GLAST were detected because of the intense sonication and solubilization for protein preparation (Suzuki et al., 2001). A standard curve was generated in increasing concentrations of a protein sample of control astrocyte cultures.

Statistical differences were determined by one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons in the case of more than two groups, or by Dunnett's test when multiple groups were compared with a single control.

To determine whether ET affects the morphology of cultured astrocytes, cells were labeled with an antibody against GFAP and rhodamine phalloidin. Intact astrocytes exhibited smooth and flat morphology and possessed apparent stress fibers of F-actin filaments (Fig. 1A). However, when treated for 10 days with 250 μM dBcAMP, a cAMP analogue, they underwent morphological changes into process-bearing stellate cells with evident GFAP immunoreactivity, and the stress fibers disappeared (Fig. 1B). These characteristics may represent differentiation corresponding to astrocytes in vivo (Schubert et al., 1997). In the presence of 100 nM ET, however, dBcAMP failed to induce this pattern of differentiation; dBcAMP did not eliminate the stress fibers; rather, it caused the swelling of cell bodies and the dilation of processes with the augmented expression of GFAP (Fig. 1C). Incidentally, ET per se caused no morphological or cytoskeletal change in intact astrocytes (data not shown).

Next, we investigated the effect of ET on glutamate uptake activity of cultured astrocytes. Treatment with 250 μM dBcAMP for 10 days significantly increased the L-[^{3}H]glutamate uptake activity (Fig. 2A). The results confirm previous reports indicating that dBcAMP enhances the expression level of glutamate transporters of primary astrocyte cultures through activation of cAMP-dependent protein kinase (PKA) (Schlag et al., 1998). In the presence of 10 or 100 nM ET, however, 250 μM dBcAMP decreased the glutamate uptake, while ET itself did not change the uptake activity in

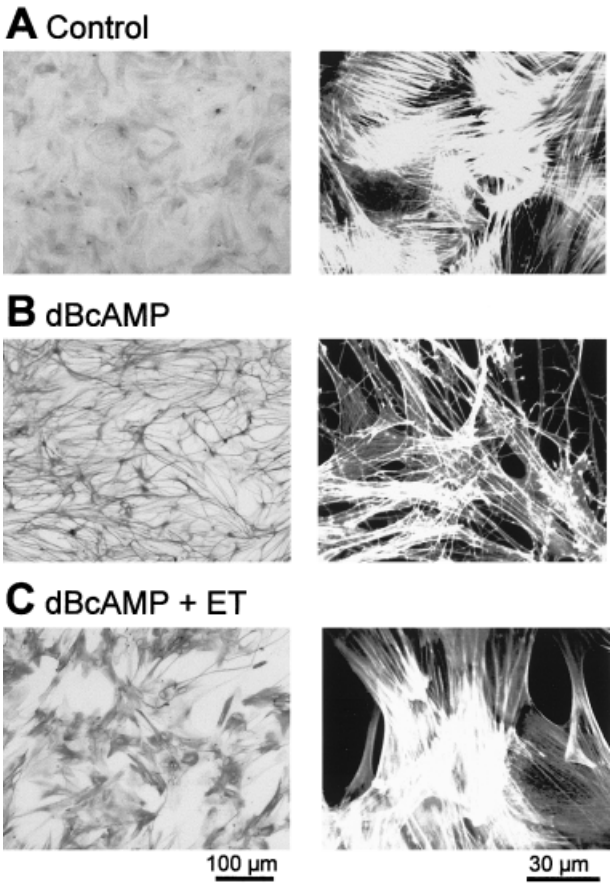

Fig. 1. Morphology and cytoskeletal organization of astrocytes treated with dBcAMP or/and endothelin (ET). Astrocytes cultured for 10 days in the absence (**A**) or presence of 250 μM dBcAMP (**B**) or a combination of 250 μM dBcAMP and 100 nM ET (**C**) were labeled with anti-GFAP antibody (left) and rhodamine-phalloidin (right).

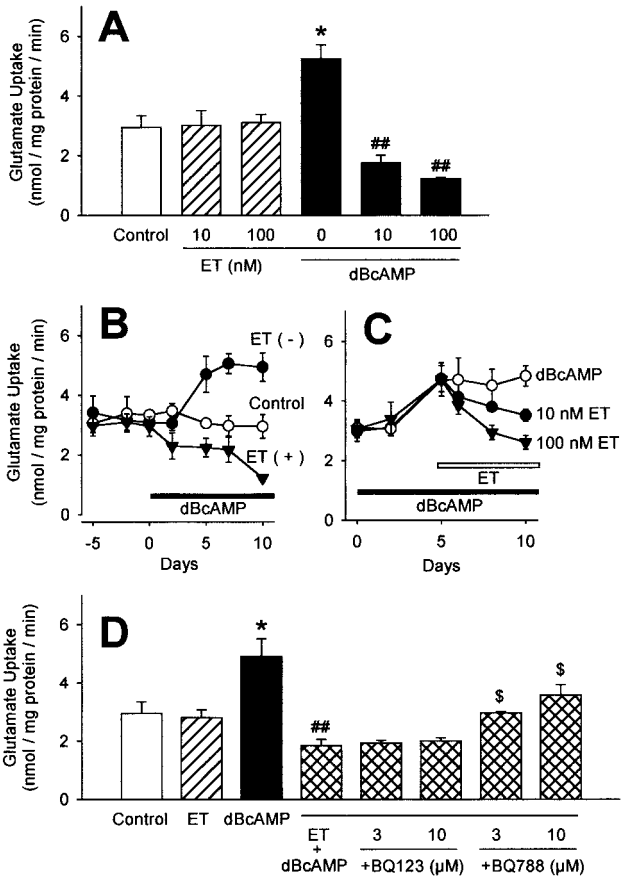

Fig. 2. dBcAMP induced an opposite effect on [^{3}H]glutamate uptake activity in endothelin (ET)-treated astrocytes. **A:** Glutamate uptake activity was measured after the treatment with dBcAMP (250 μM) or/and ET (10 or 100 nM) for 10 days. *$P < 0.05$ vs. Control; ##$P < 0.01$ vs. dBcAMP alone). **B:** Time course of the effect of 250 μM dBcAMP in the absence or presence of 100 nM ET. **C:** ET (10 or 100 nM) was added 5 days after pretreatment with 250 μM dBcAMP. **D:** Specific antagonists for ET_A and ET_B receptor (BQ123 and BQ788, respectively) were applied with 250 μM dBcAMP and 100 nM ET for 10 days. (*$P < 0.05$ vs. Control; ##$P < 0.01$ vs. dBcAMP alone; \$$P < 0.05$ vs. dBcAMP + ET). Data are means ±SEM of four cases.

undifferentiated astrocytes (Fig. 2A). Time-course analyses showed that the opposite effect of dBcAMP in ET-treated astrocytes took place within 2 days of exposure (Fig. 2B); it was also shown that ET could decrease the uptake activity that had been enhanced by dBcAMP pre-treatment (Fig. 2C).

The receptor for ET is conventionally divided into two major classes, i.e., ET_A and ET_B, both of which are expressed in astrocytes (Kasuya et al., 1994). By using specific ET_A and ET_B receptor antagonists, we determined which type of receptor is involved in the inhibition of dBcAMP-stimulated glutamate uptake. The ET effect was blocked by the ET_B receptor antagonist BQ788 not but by the ET_A receptor antagonist BQ123 (Fig. 2D), which indicates that the modulation of glutamate transport is mediated by ET_B receptor activation.

The transport activity was measured at glutamate concentrations in the range of 0 μM to 200 μM, and the data were analyzed with an Eadie–Hofstee method (Fig. 3A). Treatment with 250 μM dBcAMP for 10 days increased the average V_{max} value by 43.4% ($P < 0.01$), whereas in the presence of 100 nM ET, it decreased the V_{max} value by 41.4% ($P < 0.01$). The K_m value was unaltered in either case. Because the [^{3}H]glutamate uptake activity was almost completely abolished by 1 mM DL-threo-β-hydroxy aspartate, an inhibitor of glial glutamate transporters (6.2 ± 2.7 of control, n = 4), or by removing extracellular Na^+ (4.9 ± 0.7% of control, n = 4), the decrease in the V_{max} value suggests a reduction of the total amount of functional transporter proteins.

This possibility was addressed by Western blot analysis. The expression level of GLAST was enhanced by dBcAMP in a time-dependent manner, but it was reduced in the presence of ET (Fig. 3B). ET per se did not alter the GLAST expression. The time course of these phenomena is consistent with the result of the glutamate uptake assay. GLT-1 was also detectable in the same culture, but its expression level was considerably less than that of GLAST. Thus, we could not detect apparent changes in GLT-1 protein (data not shown; see Suzuki et al., 2001, but see also Swanson et al., 1997).

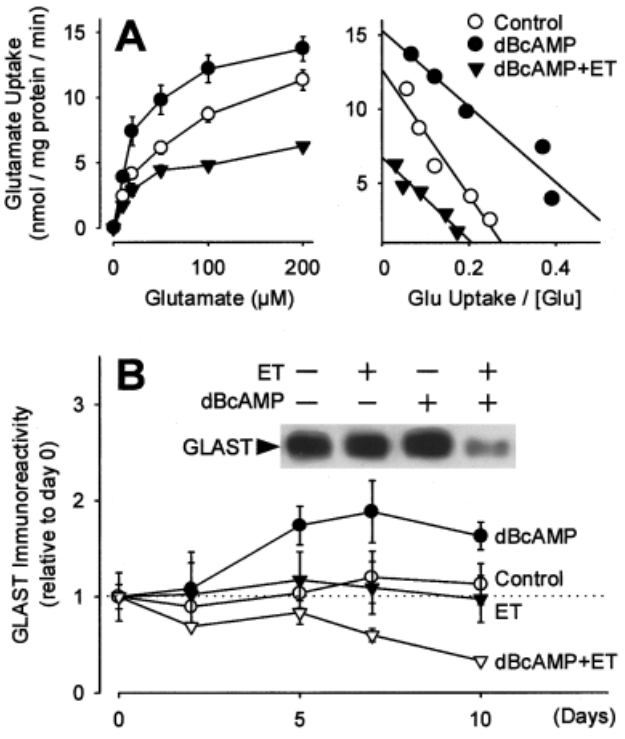

Fig. 3. cAMP stimulation causes an opposite change of GLAST expression in the presence of ET. **A:** Concentration dependence of glutamate transport activity was examined in the astrocyte treated with 250 μM dBcAMP or/and 100 nM ET for 10 days (left). Data were plotted in Eadie–Hofstee format (right). The average V_{max} values were 12.44 ± 0.59 (Control), 17.84 ± 0.62 (dBcAMP), and 7.29 ± 0.33 nmol/mg protein/min (dBcAMP + ET). The average K_m values were 36.62 ± 4.09 (Control), 28.60 ± 1.54 (dBcAMP), and 28.40 ± 4.51 μM (dBcAMP + ET). Data are means ±SEM of four cases. **B:** GLAST protein expression in astrocytes cultured in the presence of 250 μM dBcAMP or/and 100 nM ET was measured by using Western blot analysis. The level of GLAST immunoreactivity is expressed as relative to the baseline level (day 0). Equal amounts of protein (1 μg) were loaded onto each lane. Inset: Representative Western blot analysis of GLAST expression at 10 days. Data are means ±SEM of three experiments.

We have shown for the first time that ET_B receptor activation causes a reduction of GLAST expression in dBcAMP-stimulated astrocytes.

Several reports indicated that brain injuries such as trauma and ischemia elicit a twofold increase in ET (Barone et al., 1994; Uesugi et al., 1996). Our present results suggest that such an increase in ET level leads to a decrease in glutamate clearance through GLAST downregulation. Consistent with this, some studies have previously shown that traumatic and ischemic brain injuries cause a significant GLAST decrease by 30–50% (Rao et al., 1998; Yin et al., 1998). The time course of the downregulation is well correlated with brain damage after ischemia (Yin et al., 1998). Because ET receptor antagonists inhibit ischemic brain damage (Barone et al., 1995; Tatlisumak et al., 1998; Pfister et al., 2000) and GLAST-deficient mice do not exhibit spontaneous neuronal death, but do exhibit a high vulnerability to excitotoxic injury (Watase et al., 1998), we propose that ET-mediated GLAST downregulation may partly enhance excitotoxic susceptibility.

ET is assumed to trigger a substantial transformation of mature astrocytes to potentially dedifferentiated and reactive astrocytes (Hama et al., 1997; Koyama et al., 1999). Indeed, we found that ET induced the reappearance of stress fibers and the swelling of cell bodies and feet in dBcAMP-stimulated astrocytes. These characteristics are relevant to the first step of morphological changes associated with "gliosis" in brain injury (Hill et al., 1996). Although the functional significance of gliosis is not fully understood, there is considerable evidence that it produces detrimental effects, such as aberrant induction of interleukin-1β (Toulmond et al., 1996; Pearson et al., 1999) and disturbance of axonal elongation (Liuzzi and Lasek, 1987). It is therefore possible that ET exerts its pathologic effects by means of triggering gliosis as well as GLAST downregulation.

A key finding in this study is that cAMP stimulation with or without ET has bidirectional effects on GLAST activity. However, the molecular mechanisms cannot be deduced from our data alone. One of the consequences of ET_B receptor activation is an inhibition of adenylyl cyclase via G_i protein (Sakurai et al., 1990; Takigawa et al., 1995). Therefore, ET can reduce intracellular cAMP level in astrocytes. However, this signaling cascade is not likely to be involved in GLAST downregulation. Even if ET decreases the cAMP level, dBcAMP could directly activate its targets such as PKA independently of endogenous cAMP level. In addition, a combination of ET and dBcAMP reduced GLAST activity below the baseline level, whereas ET alone had no effect. This ET effect cannot be explained by cAMP signaling pathway alone. In contrast, it is interesting that ET_B receptor can also stimulate mitogen-activated protein kinase (MAPK) cascade via G_q protein (Cazaubon et al., 1997). A reciprocal interaction between MAPK cascade and cAMP signaling has been argued intensively in many types of cells (Hordijk et al., 1994; Vossler et al., 1997; Yao et al., 1998; Hoffmann et al., 1999). In astrocytes, MAPK activation, which leads to diverse events, including mitogenesis and growth factor synthesis (Kasuya et al., 1994; Biesiada et al., 1996; Pedram et al., 1998), is inhibited by increasing cAMP level (Kurino et al., 1996). Therefore, it is plausible that the cross-talk between ET_B receptor downstream and cAMP signaling contributes to dBcAMP-induced opposite regulation of GLAST. To date, very little is known about the regulatory factors of GLAST expression or the promoter region of GLAST gene. Further elucidation of the mechanisms underlying ET-mediated, bidirectional GLAST modulation would provide a novel insight into GLAST regulatory system.

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