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

SIGERU MATSUURA, YUJI IKEGAYA, MAKI K. YAMADA, NOBUYOSHI NISHIYAMA, AND NORIO MATSUKI
Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

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endothelin receptor; Na⁺-dependent glutamate transporter; astrocyte; glial cell; GLT-1

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 [³H]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 ETB receptor, but not of ETA receptor. ET per se was virtually ineffective. Eadie–Hofstee analysis demonstrated that dBcAMP increased the Vmax 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 Km 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-
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-angioblin acid protein (GFAP) antibody (Amersham, Buckinghamshire, UK), and L-[3H]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 250g for 5 min, and the pellet was re-suspended in Eagle's minimum essential medium with 10% fetal bovine serum (FBS) and placed into 75-cm² culture flasks at a density of 1 × 10⁶ cells/cm². Cultures were maintained in a humidified, 5% CO₂ 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 × 10⁴ cells/cm². 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-[3H]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 Na₂HPO₄, 4.2 mM NaHCO₃, 0.44 mM KH₂PO₄, 5.37 mM KCl, 1.26 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, and 2 mM glucose at pH 7.4, and then exposed to [3H]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 800g 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 (1,500) 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-[3H]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...
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., ETA and ETB, both of which are expressed in astrocytes (Kasuya et al., 1994). By using specific ETA and ETB 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 ETB receptor antagonist BQ788 not but by the ETA receptor antagonist BQ123 (Fig. 2D), which indicates that the modulation of glutamate transport is mediated by ETB 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 [3H]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). 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).
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 (Toumond 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<sub>B</sub> receptor activation is an inhibition of adenylyl cyclase via G<sub>i</sub> 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<sub>B</sub> receptor can also stimulate mitogen-activated protein kinase (MAPK) cascade via G<sub>i</sub> protein (Cazabon 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<sub>B</sub> 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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