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Glutamate transporter function of rat hippocampal astrocytes is impaired following the global ischemia

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Astroglial glutamate transporters, GLT-1 and GLAST, play an essential role in removing released glutamate from the extracellular space and are essential for maintaining a low concentration of extracellular glutamate in the brain. It was hypothesized that impaired function of glial glutamate transporters induced by transient global ischemia may lead to an elevated level of extracellular glutamate and subsequent excitotoxic neuronal death. To test this hypothesis, in the present study, we performed whole-cell patch-clamp recording of hippocampal CA1 astrocytes in control or postischemic slices, and measured glutamate transporter activity by recording glutamate-evoked transporter currents. Six to 24 h after global ischemia, maximal amplitude of glutamate transporter currents recorded from postischemic CA1 astrocytes was significantly reduced. Western blotting analysis indicated that transient global ischemia decreased the protein level of GLT-1 in the hippocampal CA1 area without affecting GLAST protein level. Further real-time quantitative RT-PCR assays showed that global ischemia resulted in a decrease in GLT-1 mRNA level of hippocampal CA1 region. Global ischemia-induced reduction in GLT-1 expression and glutamate transporter function of CA1 astrocytes precedes the initiation of delayed neuronal death in CA1 pyramidal layer. The present study provides the evidence that transient global ischemia downregulates glutamate transporter function of hippocampal CA1 astrocytes by decreasing mRNA and protein levels of GLT-1.

Keywords: Ischemia; CA1 astrocyte; Glutamate transporter; Transporter current; GLT1 transporter; GLAST transporter

Introduction

Sodium-dependent glutamate transporters expressed in astroglial cells and neurons are responsible for removing released glutamate from the extracellular space (for review see Anderson and Swanson, 2000; Danbolt, 2001; Gadea and Lopez-Colome, 2001). To date, five subtypes of high-affinity Na^+-dependent glutamate transporters, GLAST/EAAT1, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5, have been cloned from the brain (Arriza et al., 1997; Fariman et al., 1995; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). Immunocytochemical studies demonstrated that GLAST and GLT-1 are mainly expressed in astrocytes, with GLAST predominating in the cerebellum and GLT-1 predominating in the cerebral cortex and hippocampus (Lehre and Danbolt, 1998; Lehre et al., 1995). EAAC1 is found in neurons throughout the brain, whereas the expressions of EAAT4 and EAAT5 are largely confined to cerebellar Purkinje neurons and retina, respectively (Arriza et al., 1997; Fariman et al., 1995; Gadea and Lopez-Colome, 2001). Knockout experiments using antisense oligonucleotides or transgenic mice showed that the loss of astroglial transporters, GLT-1 and GLAST, led to a tonic increase in extracellular glutamate concentration and subsequent neurodegeneration (Mitani and Tanaka, 2003; Rothstein et al., 1996). It has been reported that transgenic mice lacking GLT-1 showed spontaneous epileptic seizures, selective neurodegeneration in the hippocampus, and exacerbation of acute cortical injury (Tanaka et al., 1997). Mutant mice deficient in GLAST exhibited an increased susceptibility to cerebellar injury and motor discoordination (Watase et al., 1998). In contrast, EAAC1 knockout mice showed a reduction in locomotor activity without neurodegeneration in the brain (Peghini et al., 1997). Thus, GLT-1 and GLAST play an essential role in removing released glutamate and are essential for maintaining a low concentration of extracellular glutamate in the brain.

A large body of evidence indicates that glutamate excitotoxicity plays a major role in the molecular mechanism underlying delayed

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neuronal death following the brain ischemia (for review, see Choi, 1994; Lipton, 1999). Considering the physiological importance of GLT-1 and GLAST in removing extracellular glutamate, it is reasonable to hypothesize that impaired function of astroglial glutamate transporters induced by the ischemic assault could lead to an elevated level of extracellular glutamate, which subsequently may potentiate NMDA and AMPA receptor-mediated postsynaptic excitation and result in excitotoxic neuronal death (Danbolt, 2001). Transporter-mediated entry of each glutamate anion into the cell is accompanied by the cotransport of two or three Na^+ with one H^+, and the countertransport of one K^+. Because of the translocation of net positive charge during each transport cycle, glutamate uptake by GLT-1 or GLAST transporter is an electrogenic process (Levy et al., 1998; Seal and Amara, 1999; Wadiche et al., 1995). Therefore, electrophysiological recording of transporter currents has been used to study the function of astroglial glutamate transporters (Bergles and Jahr, 1997; Brew and Attwell, 1987; Trotti et al., 2001). To investigate possible ischemia-induced alteration in glutamate transporter function, we recorded glutamate-evoked transporter currents from CA1 astrocytes of the stratum radiatum in control or postischemic hippocampal slices. Our results indicate that glutamate transporter activity of hippocampal CA1 astrocytes is significantly reduced following transient global ischemia. Further Western blotting analysis and real-time quantitative RT-PCR assay suggest that global ischemia downregulates glutamate transporter function of hippocampal CA1 astrocytes by decreasing mRNA and protein levels of GLT-1.

Materials and methods

Global ischemia in the rat

Transient global ischemia of Sprague–Dawley rats, 8 to 9 weeks old, was produced by performing four-vessel occlusion (Pulsinelli and Buchan, 1988; Pulsinelli et al., 1982). Briefly, on the first day, both vertebral arteries were exposed and electrocauterized under an anesthesia with halothane. Common carotid arteries were also exposed following a transverse neck incision, and a small loop of elastic tube was placed loosely around each vessel. On the next day, common carotid arteries were occluded for 20 min by clamping the vessels with atraumatic aneurysm clips. Body temperature of the rat was maintained at 37°C. For sham-operated rats, all procedures were performed exactly as for ischemic animals with the exception that carotid arteries were not clamped. Global ischemia-induced neurodegeneration was assessed by histological examination of brain sections. Briefly, rats were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed brains were then sectioned coronally (20 μm thick), stained with thionine (0.25%) and neuronal damage was evaluated under a light microscope.

Whole-cell patch-clamp recordings in brain slices

Control or postischemic rats were terminally anesthetized and decapitated. The whole brain was quickly removed, and 200-μm-thick transverse hippocampal slices were prepared by using a vibratome slicer (VT 1000S, Leica) in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 5, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2.5, NaH_2PO_4 1, and glucose 11. Before being used for electrophysiological recordings, brain slices were transferred to a holding chamber where they were submerged in the ACSF gassed with 95% O_2/5% CO_2 for 1 h.

Following the recovery period, a single hippocampal slice was transferred to a recording chamber and superfused continuously with ACSF pregassed with 95% O_2/5% CO_2. Cell body of CA1 astrocyte located in the stratum radiatum was visualized with a 40× water immersion objective (Zeiss) using an Axioskop (Zeiss) microscope equipped with differential interference contrast (DIC) optics and infrared light-sensitive camera (Newvicon C2400-07C, Hamamatsu) (Bergles and Jahr, 1997). In the presence of antagonists of $GABA_A$ receptor (200 μM picrotoxin), AMPA/kainate receptors (20 μM NBQX), NMDA receptor (20 μM D-CPP), and group I/II metabotropic glutamate receptors (400 μM MCPG), transporter current was evoked by applying glutamate to astrocytes using the puffer ejection (Picospritzer, General Valve) from blunt micropipettes (diameter = ~10 μm). Patch pipets were filled with the solution containing (mM):70 KCl, 60 KF, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 2 ATP and 0.3 GTP, pH = 7.2. Glutamate-evoked transporter currents recorded by the patch-clamp amplifier (Axopatch-200B, Axon Instruments) were filtered, digitized (Digidata 1200, Axon Instruments) and stored for a later analysis. Series resistance was usually <10 MΩ, and the compensation circuitry of the amplifier was used to minimize the series resistance error. Liquid junction potentials were corrected as described previously (Li et al., 2001). Holding potentials, data acquisition and analysis were controlled by an online IBM-PC compatible computer programmed with software pCLAMP 7.0 (Axon Instruments). Prism program (GraphPad Software) was used to analyze the dose–response curve. All the whole-cell patch-clamp recordings were performed at room temperature (23–25°C).

Putative astrocytes in brain slice were double-labeled by combining whole-cell recordings using the pipet solution containing 0.3% Lucifer Yellow (Sigma) with glial fibrillary acidic protein (GFAP) immunofluorescence staining. Briefly, hippocampal slices were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Then, slices were immersed in 10% normal goat serum and 0.1% Triton X-100 for 1 h. Subsequently, slices were incubated with rabbit anti-GFAP polyclonal antibody (Chemicon) for 72 h at 4°C. Following the wash, slices were incubated with PBS containing Texas Red-conjugated goat anti-rabbit IgG (Vector). GFAP-positive astrocytes were viewed and photographed under a Zeiss Axioskop microscope equipped with Epifluorescence system.

Western blotting analysis

To monitor the protein expression of GLAST or GLT-1, rats were sacrificed at designated times after ischemic insult. Hippocampal CA1 region was dissected out and homogenized in a 10-mM Tris buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM dithiothreitol, and a cocktail of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 5 μg/ml aprotinin). Following the centrifugation at 11,000 × *g* for 5 min, the resulting pellet was dissolved in gel loading buffer (31.2 mM Tris, 1% sodium dodecyl sulfate (SDS), 5% glycerol, and 2.5% β-mercaptoethanol) and centrifuged at 20,000 × *g* for 30 min. The supernatant was used as the source of proteins, and protein concentration was determined with the Coomassie blue protein assay. For gel electrophoresis, each lane was loaded with approximately 40 μg of protein. After separation by 10% SDS-polyacrylamide gel electrophoresis, proteins on the gel were electrotransferred to a nitrocellulose filter (Hybond; Amersham).