Biochemical and Biophysical Research Communications 393 (2010) 514–518

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PI3K/Akt/mTOR signaling regulates glutamate transporter 1 in astrocytes

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ARTICLE INFO

Article history:
Received 3 February 2010
Available online 10 February 2010

Keywords:
Astrocyte
Astrocyte-defined medium (ADM)
Glutamate uptake
Glutamate transporter 1 (GLT1)
Mammalian target of rapamycin (mTOR)
Rapamycin

ABSTRACT

Reduction in or dysfunction of glutamate transporter 1 (GLT1) is linked to several neuronal disorders such as stroke, Alzheimer's disease, and amyotrophic lateral sclerosis. However, the detailed mechanism underlying GLT1 regulation has not been fully elucidated. In the present study, we first demonstrated the effects of mammalian target of rapamycin (mTOR) signaling on GLT1 regulation. We prepared astrocytes cultured in astrocyte-defined medium (ADM), which contains several growth factors including epidermal growth factor (EGF) and insulin. The levels of phosphorylated Akt (Ser473) and mTOR (Ser2448) increased, and GLT1 levels were increased in ADM-cultured astrocytes. Treatment with a phosphatidylinositol 3-kinase (PI3K) inhibitor or an Akt inhibitor suppressed the phosphorylation of Akt (Ser473) and mTOR (Ser2448) as well as decreased ADM-induced GLT1 upregulation. Treatment with the mTOR inhibitor rapamycin decreased GLT1 protein and mRNA levels. In contrast, rapamycin did not affect Akt (Ser473) phosphorylation. Our results suggest that mTOR is a downstream target of the PI3K/Akt pathway regulating GLT1 expression.

Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family and is a key regulator of cell growth, proliferation, metabolism, and survival [1]. mTOR plays an evolutionarily conserved role in integrating the signals from growth factors [2]. Among various pathways upstream of mTOR, PI3K/Akt signaling is considered the most important [2]. Growth factors including epidermal growth factor (EGF) and insulin activate the mTOR pathway via PI3K/Akt signaling and stimulate cell cycle progression and proliferation [3–5]. Regulation of the mTOR pathway plays an important physiological and pathological role in the central nervous system (CNS).

Glutamate, a principal excitatory amino acid mediating fast neurotransmission in the CNS, plays a central role in normal brain functions including cognition, memory, and learning. However, excessive elevation of the extracellular glutamate concentration mediates excitotoxicity and causes neuronal cell death [6]. There is no enzymatic system available for metabolizing glutamate in the extracellular space; the only way to maintain glutamate homeostasis is through glutamate uptake via glutamate transporters [7].

Five glutamate transporter subtypes expressed in neurons or glia, including glutamate aspartate transporter (GLAST, also known as EAAT1), glutamate transporter 1 (GLT1, also known as EAAT2), EAAC1, EAAT4, and EAAT5, have been identified and characterized. GLT1, predominantly located on astrocytes, is responsible for up to 90% of glutamate clearance in adult brain tissue [7].

Reductions in or dysfunctions of GLT1 have been documented in several neurological disorders including stroke [8], Alzheimer's disease [9], and amyotrophic lateral sclerosis [10]. These findings emphasize the importance of GLT1 in astrocytes for normal brain function.

In the present study, we prepared astrocytes incubated in astrocyte-defined medium (ADM), which contained a number of supplementary agents including the growth factors EGF, bFGF, and insulin [11,12]. It has been demonstrated that GLT1 expression is increased by EGF, and EGF–Akt signals regulate GLT1 expression [13]. Because it is a well-known substrate of Akt, mTOR has been proposed to have a role in GLT1 regulation.

We therefore investigated whether mTOR is involved in GLT1 regulation using ADM-cultured astrocytes.

Materials and methods

Astrocyte cultures. The use of experimental animals in this study was conducted in accordance with the Ethical Guidelines of the Kyoto University Animal Experimentation Committee. Primary astrocyte cultures were obtained from postnatal day 1 (P1) Wistar rat cortex (Nihon SLC, Shizuoka, Japan). Briefly, dissociated cells were seeded into 75-cm^2 tissue culture flasks and incubated for 20–24 days in Eagle's minimum essential medium (EMEM) supple-

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doi:10.1016/j.bbrc.2010.02.038

mented with 10% FBS and 50 μg/ml kanamycin at 37 °C in a 5% CO_2/95% air humidified incubator. Flasks were shaken at 400 rpm (10 min, 37 °C) and then at 220 rpm (15 h, 37 °C). Astrocyte cultures were detached with solution minimal essential medium (SMEM) containing 0.25 μg/ml trypsin and were cultured in Dulbecco's modified Eagle's medium (DMEM) (10% FBS and 50 μg/ml kanamycin) at 2.5×10^4 cells/cm^2. Astrocyte-defined medium (ADM) was formulated as follows: DMEM was supplemented with transferrin (50 μg/ml), sodium selenite (5.2 ng/ml), fibronectin (1.5 μg/ml), heparin sulfate (0.5 μg/ml), EGF (10 ng/ml), bFGF (5 ng/ml), insulin (5 μg/ml), and kanamycin (50 μg/ml) [12]. Cells in the culture were shown to be astrocytes with a purity of 97 ± 2% after characterization by immunostaining using a primary anti-GFAP antibody.

L-[^{3}H]Glutamate uptake assay. Astrocyte cultures grown in 24-well plates were used in an L-[^{3}H]glutamate (GE Healthcare, Buckinghamshire, UK) uptake assay as described previously [14]. Briefly, cells were incubated with Na^+ or Na^+-free uptake buffer containing 100 μM glutamate and 1 μCi/ml L-[^{3}H]glutamate for 10 min. L-[^{3}H]Glutamate uptake was stopped by washing with ice-cold Dulbecco's phosphate buffered saline (DPBS). The radioactivity of the cell lysate was measured using a liquid scintillation counter; a fraction of the remaining lysate was used for protein concentration determination. After subtraction of the basal L-[^{3}H]glutamate uptake amount (L-[^{3}H]glutamate uptake value in Na^+-free buffer), the L-[^{3}H]glutamate uptake values in the control and experimental groups were normalized by protein concentration and plotted as the percentage of control L-[^{3}H]glutamate uptake values.

Western blot analysis. Western blot analysis was conducted as previously described [15] using rabbit polyclonal anti-Akt (Cell Signaling Danvers, MA), rabbit polyclonal anti-p-Akt (Ser473) (Cell signaling), rabbit polyclonal anti-mTOR (Cell signaling), rabbit polyclonal anti-p-mTOR (Ser2448) (Cell signaling), rabbit polyclonal anti-GLT1 (Santa Cruz), rabbit polyclonal anti-GLAST (Santa Cruz) or mouse monoclonal anti-GAPDH (Ambion, Austin, TX).

Real-time PCR. Real-time PCR (RT-PCR) was conducted as previously described [16]. The primers used were as follows: rat GLT1 forward: 5′-GTT CAA GGA CGG GAT GAA TGT CTT A-3′ and reverse: 5′-CAT CAG CTT GGC CTG CTC AC-3′; and rat cyclophilin A forward: 5′-GGC AAA TGC TGG ACC AAA CAC-3′ and reverse: 5′-AAA CGC TCC ATG GCT TCC AC-3′. Each sample was run in triplicate and normalized to the relative amplification of rat cyclophilin A.

Statistical analysis. Values were expressed as the means ± SE of at least 3 independent experiments. Differences between means were determined with Student's *t*-test or a one-way ANOVA followed by Newman–Keuls' multiple comparison test using GraphPad Prism Software 4.03. Differences were considered significant at $P < 0.05$.

Results

ADM induces GLT1 expression and Akt and mTOR phosphorylation

GLT1 protein was detectable in astrocytes incubated in DMEM with 10% FBS (control; CTL). When astrocytes were incubated in astrocyte-defined medium (ADM) as previously reported [11,12], GLT1 expression was upregulated. Western blot analysis showed that GLT1 expression increased in a time-dependent manner in ADM and was significantly higher than expression in the CTL after a one-day incubation. In contrast, GLAST, another glutamate transporter mainly located on astrocytes, was not affected (Fig. 1A and B).

To determine whether the change in GLT1 expression affected glutamate uptake capacity in ADM-cultured astrocytes, an L-[^{3}H]glutamate uptake assay was performed. Astrocytes incubated in ADM showed a time-dependent increase in L-[^{3}H]glutamate uptake. Pre-incubation with dihydrokainic acid (DHK, 1 mM, 3 min), a

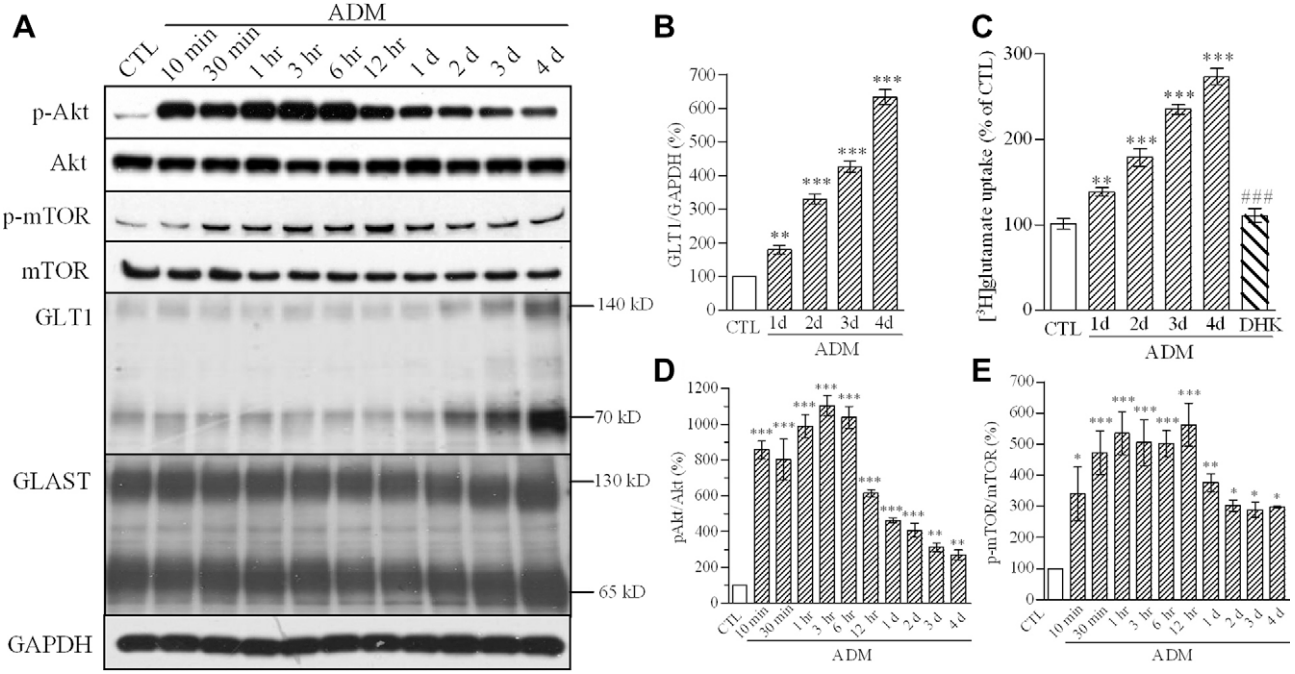

Fig. 1. ADM induces GLT1 expression and Akt (Ser473) and mTOR (Ser2448) phosphorylation. (A, B, D, E) Western blot analysis showed that a four days of ADM culturing induced GLT1 expression, Akt phosphorylation at Ser473 and mTOR phosphorylation at Ser2448 in a time-dependent manner. (C) ADM culturing induced a time-dependent (from day 1 to day 4) increase in glutamate uptake in astrocytes, and this uptake was almost totally abolished by pre-treatment (3 min) with a specific GLT1 inhibitor DHK (1 mM). Data represent the means ± SE of 4–6 independent experiments. $^{***}P < 0.001$ versus CTL (control; DMEM with 10% FBS); $^{\#\#\#}P < 0.001$ versus ADM (4 days). DHK: dihydrokainic acid.