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RE:

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

Dear Editor,

We would be very pleased if you would consider our manuscript for publication in *Biochemical and Biophysical Research Communications*.

Below is a brief summary of the background and purpose of our study.

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.

Mammalian target of rapamycin (mTOR) is a key regulator of protein translation and is responsive to a diverse group of cellular signals including growth factors.

We therefore hypothesized that growth factor-induced activation of mTOR would upregulate GLT1.

We prepared astrocytes cultured in astrocyte-defined medium (ADM), which contains several growth factors including epidermal growth factor (EGF). As a result of culturing the cells in this medium, the levels of phosphorylated Akt (Ser473) and mTOR (Ser2448) increased, and GLT1 expressions and activities were increased in ADM-cultured astrocytes. Treatment of the cells 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.

Our results suggest that mTOR is a downstream target of the PI3K/Akt pathway regulating GLT1 expression.

This is the first report showing that mTOR is downstream of PI3K/Akt in the pathway regulating GLT1 expression. We believe that this manuscript is suitable for publication in *Biochemical and Biophysical Research Communications*.

Yours sincerely,

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JAPAN

Title

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

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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 insulin and 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.

Keywords

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Mammalian target of rapamycin (mTOR)

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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 [3,4,5]. 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-cm² tissue culture flasks and incubated for 20-24 days in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS and 50 µg/ml kanamycin at 37°C in a 5% CO₂/95% air humidified incubator. Flasks were shaken at 400 rpm (10 min, 37°C) and then at 220 rpm (15 hr, 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/cm². 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 $97 \pm 2\%$ after characterization by immunostaining using a primary anti-GFAP antibody.

L-[³H]glutamate uptake assay. Astrocyte cultures grown in 24-well plates were used in an L-[³H]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-[³H]glutamate for 10 minutes. L-[³H]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-[³H]glutamate uptake amount (L-[³H]glutamate uptake value in Na⁺-free buffer), the L-[³H]glutamate uptake values in the control and experimental groups were normalized by protein concentration and plotted as the percentage of control L-[³H]-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 (Ser 473) (Cell signaling), rabbit polyclonal anti-mTOR (Cell signaling), rabbit polyclonal anti-p-mTOR (Ser 2448) (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 mean \pm S.E. of at least three 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, 1B).

To determine whether the change in GLT1 expression affected glutamate uptake capacity in ADM-cultured astrocytes, an L-[³H]glutamate uptake assay was performed. Astrocytes incubated in ADM showed a time-dependent increase in L-[³H]glutamate uptake. Pre-incubation with dihydrokainic acid (DHK, 1 mM, 3 min), a specific GLT1 inhibitor, almost totally abolished the four-day ADM-induced increase in L-[³H]glutamate uptake (Fig. 1C), indicating that the ADM-induced increase in L-[³H]glutamate uptake was caused by the upregulation of GLT1.

To determine whether the Akt/mTOR pathway was activated in ADM-cultured astrocytes, we examined the phosphorylation level of Akt at Ser473 and the phosphorylation level of mTOR at Ser2448. The results showed that Akt was phosphorylated immediately after the medium was replaced with ADM, and the phosphorylation reached its peak 6 hr after the medium replacement. This peak was followed by a gradual decrease, although the increased phosphorylation was sustained even after four days (Fig. 1A, 1D).

Subsequent phosphorylation of mTOR was found after the beginning of Akt phosphorylation, and mTOR phosphorylation reached its peak 12 hr after the medium replacement. As for Akt phosphorylation, this peak was followed by a gradual decrease, although the increased phosphorylation was sustained even after four days (Fig. 1A, 1E).

GLT1 upregulation was found after a one-day incubation, suggesting that Akt/mTOR signaling might lead to GLT1 protein synthesis (Fig. 1A).

mTOR is a downstream target of PI3K/Akt in the pathway regulating GLT1 expression

To determine whether the Akt/mTOR pathway is involved in GLT1 expression, the effects of PI3K inhibition on GLT1 expression were examined. Treatment with a selective PI3K inhibitor LY294002 (from 0.3 μ M to 10 μ M) for 4 days decreased Akt phosphorylation (Ser473) in a concentration-dependent manner in four-day ADM-cultured astrocytes (Fig. 2A, 2B). In addition, mTOR phosphorylation (Ser2448), and GLT1 protein levels were also suppressed by LY294002, indicating that mTOR is a downstream target of PI3K (Fig. 2A, 2C, 2D).

For the Akt inhibition, we selected Akt inhibitor VIII since it selectively blocks basal and stimulated phosphorylation of Akt1/Akt2. Treatment with Akt inhibitor VIII (from 0.03 μ M to 1 μ M) for 4 days also decreased Akt phosphorylation (Ser473), mTOR phosphorylation (Ser2448), and GLT1 protein levels in a concentration-dependent manner in four-day ADM-cultured astrocytes (Fig. 2A, E-G).

It is well known that PI3K phosphorylates Akt. Based on these data, it is hypothesized that mTOR is a downstream target of Akt.

Phosphorylation of mTOR is involved in GLT1 expression

Rapamycin, an antifungal and immunosuppressant agent, complexes with FKBP-12 and binds mTOR and has been reported to inhibit the activity of mTOR and mTOR phosphorylation at Ser2448. Treatment with rapamycin (from 1 nM to 30 nM) for 4 days decreased mTOR phosphorylation (Ser2448) and GLT1 protein levels in four-day ADM-cultured astrocytes (Fig. 3A-3D). However, rapamycin had no effect on Akt phosphorylation (Ser473) (Fig. 3A-3D). Based on these data, it is hypothesized that PI3K-Akt is upstream of mTOR.

To demonstrate that the ADM-induced increase in GLT1 levels is caused by the protein synthesis and not by the attenuation of degradation and that mTOR plays a key role in this cascade, we evaluated GLT1 mRNA levels by real-time PCR. Incubation in ADM caused an increase in GLT1 mRNA levels, and rapamycin significantly reduced the ADM-induced increase in GLT1 mRNA (Fig. 3G). These results suggest that mTOR activation is involved in the increase of GLT1 mRNA.

We next investigated whether the mTOR inhibition-induced decrease in GLT1

expression affected the glutamate uptake capacity of four-day ADM-cultured astrocytes by performing a L-[³H]glutamate uptake assay. Treatment with rapamycin decreased the ADM-induced increase in glutamate uptake in a time- and concentration-dependent manner (Fig. 3E, 3F). These results suggest that glutamate uptake activity is correlated with GLT1 protein levels.

Based on these results, it is hypothesized that mTOR is downstream of the PI3K/Akt pathway that regulates GLT1 protein levels and activity.

Discussion

In this study, we revealed that mTOR is a downstream target of Akt in the pathway regulating GLT1 expression. This conclusion is based on the following results obtained from experiments with ADM-cultured astrocytes: (1) ADM induced Akt/mTOR pathway activation and GLT1 expression; (2) Akt inhibition reduced mTOR phosphorylation and GLT1 expression; and (3) mTOR inhibition reduced GLT1 expression and glutamate uptake capacity but did not affect Akt phosphorylation.

mTOR is responsive to a diverse group of cellular signals including growth factors, and mTOR is a key regulator of protein translation. Growth factors or related hormones activate receptor tyrosine kinases and G protein coupled receptors, and several signal transduction pathways are subsequently activated [17]. PI3K/Akt pathway and the Ras/Erk (extracellular signal regulated kinase) pathway stimulate mTOR [18]. Growth factors and insulin in ADM would activate these signal cascade.

Activated mTOR subsequently signals to various components of the translation initiation machinery through direct or indirect phosphorylation. mTOR-mediated phosphorylation activates ribosomal S6 kinase, results in its dissociation from eukaryotic initiation factor complex 3 (eIF3) [19]. Subsequently 4E-binding protein 1 (4E-BP-1) dissociates from the cap complex to promote protein translation [19]. GLT1 upregulation might be mediated via these signals, although the downstream transcription factor(s) binding the GLT1 promoter need to be identified in future experiments.

Cells have several independent mechanisms, among them mTOR-mediated regulation of translation initiation. Interestingly, GLAST expression was not altered by mTOR inhibition. GLT1 and GLAST are regulated by different signals.

It has been reported that mTOR signaling is important for astrocyte survival during ischemia. During oxygen-glucose depletion, mTOR signaling is impaired in astrocytes, resulting in a defect in Bad phosphorylation and in the expression of anti-apoptotic factors Bcl-2 and Bcl-xl, leading to dysfunction or death of astrocytes [20]. Normal GLT1 expression and glutamate uptake capacity are also impaired during ischemia [21].

Oxygen-glucose depletion or ischemia might impair mTOR signaling, and as a consequence, GLT1 expression might be downregulated.

Conversely, activation of mTOR signaling would upregulate GLT1, which could reduce excessive extracellular glutamate to decrease the size of ischemic regions or the impact of neurodegenerative diseases.

Our novel findings that mTOR is involved in the PI3K/Akt-induced upregulation of GLT1 contribute to the understanding of the pathogenesis of ischemia and neurodegenerative diseases and may be important in developing therapeutic strategies against these conditions.

In conclusion, this is the first report showing that mTOR is downstream of PI3K/Akt in the pathway regulating GLT1 expression, and our finding reveals a new mechanism underlying GLT1 regulation in astrocytes.

Legend

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 mean \pm S.E. of 4-6 independent experiments. *** $P < 0.001$ versus CTL (control; DMEM with 10% FBS); ### $P < 0.001$ versus ADM (4 d). DHK: dihydrokainic acid.

Fig. 2. mTOR is a downstream target of PI3K/Akt in the pathway regulating GLT1 expression.

(A-D) Four-day treatment with a PI3K inhibitor, LY294002, significantly decreased Akt phosphorylation at Ser473, mTOR phosphorylation at Ser2448, and GLT1 expression in four-day ADM-cultured astrocytes in a concentration-dependent manner (from 0.3 μ M to 10 μ M). (A, E-G) Four-day treatment with an Akt inhibitor, Akt inhibitor VIII, significantly decreased Akt phosphorylation at Ser473, mTOR phosphorylation at Ser2448, and GLT1 expression in four-day ADM-cultured astrocytes in a concentration-dependent manner (from 0.03 μ M to 1 μ M). Data represent the mean \pm S.E. of 4 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus CTL (control; ADM). Akt inhibitor: Akt inhibitor VIII.

Fig. 3. Phosphorylation of mTOR is involved in GLT1 expression.

(A-D) Four-day treatment with the mTOR inhibitor rapamycin decreased mTOR phosphorylation at Ser2448 and GLT1 expression in a concentration-dependent manner (from 1 nM to 30 nM). Rapamycin had no effect on Akt phosphorylation at Ser 473 in four-day ADM-cultured astrocytes. (E) Treatment with rapamycin (30 nM) decreased the ADM-induced increase in glutamate uptake in a time-dependent manner (from day 1 to day 4). (F) Four-day treatment with rapamycin decreased the ADM-induced increase

in glutamate uptake in a concentration-dependent manner (from 1 nM to 30 nM) in four-day ADM-cultured astrocytes. (G) Four-day treatment with rapamycin (30 nM) decreased the ADM-induced increase in GLT1 mRNA levels in four-day ADM-cultured astrocytes. Data represent the mean \pm S.E. of 4-6 independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 versus CTL (control; ADM); ### P < 0.001 versus CTL (control; DMEM with 10% FBS).

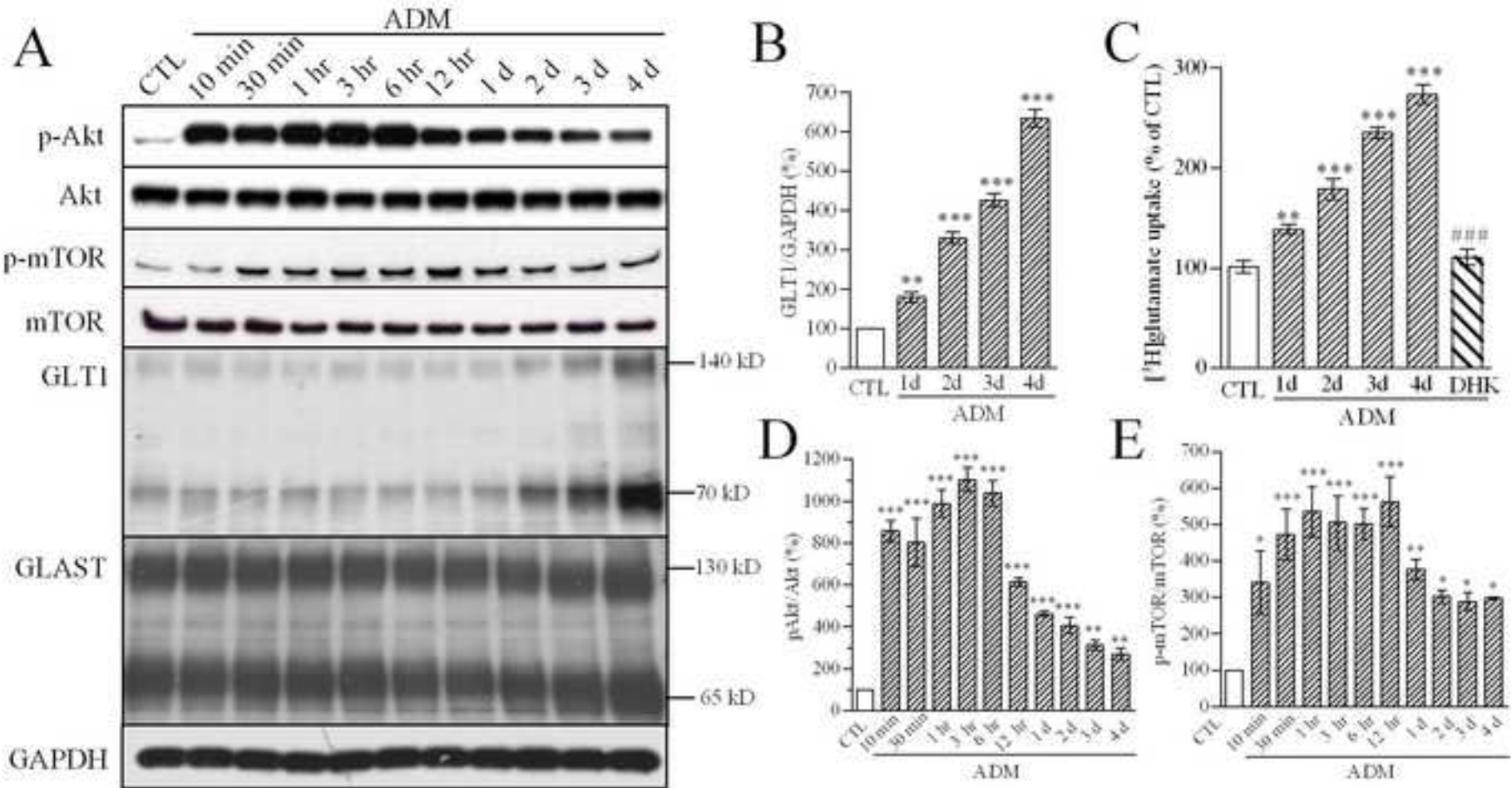
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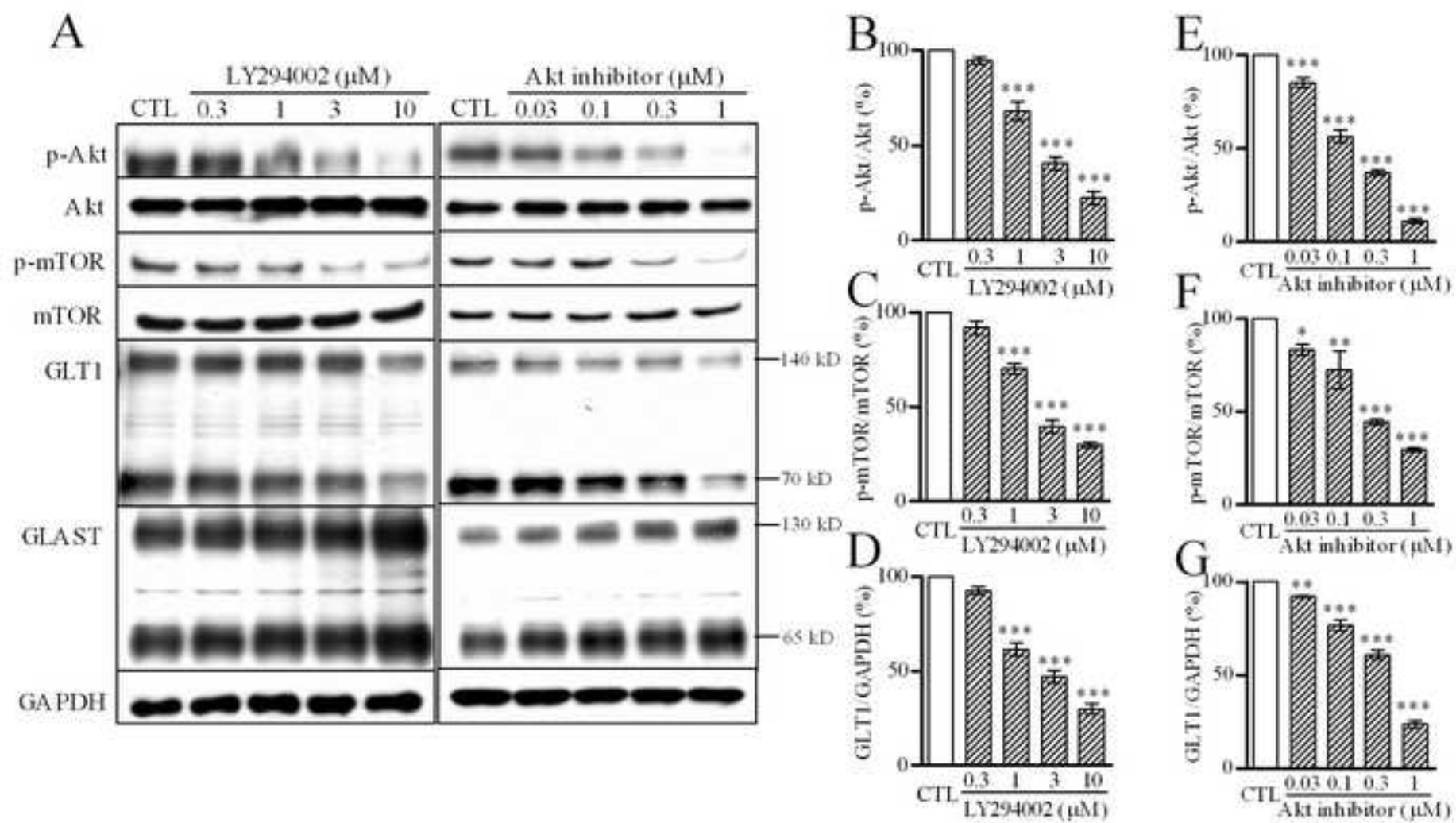


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