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Possible activation by the green tea amino acid theanine of mammalian target of rapamycin signaling in undifferentiated neural progenitor cells *in vitro*

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We have shown marked promotion of both proliferation and neuronal differentiation in pluripotent P19 cells exposed to the green tea amino acid theanine, which is a good substrate for SLC38A1 responsible for glutamine transport. In this study, we evaluated the activity of the mammalian target of rapamycin (mTOR) kinase pathway, which participates in protein translation, cell growth and autophagy in a manner relevant to intracellular glutamine levels, in murine neural progenitor cells exposed to theanine. Exposure to theanine promoted the phosphorylation of mTOR and downstream proteins in neurospheres from embryonic mouse neocortex. Although stable overexpression of SLC38A1 similarly facilitated phosphorylation of mTOR-relevant proteins in undifferentiated P19 cells, theanine failed to additionally accelerate the increased phosphorylation in these stable transfectants. Theanine accelerated the formation of neurospheres from murine embryonic neocortex and adult hippocampus, along with facilitation of both 5-bromo-2'-deoxyuridine incorporation and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction in embryonic neurospheres. In embryonic neurospheres previously exposed to theanine, a significant increase was seen in the number of cells immunoreactive for a neuronal marker protein after spontaneous differentiation. These results suggest that theanine activates the mTOR signaling pathway for proliferation together with accelerated neurogenesis in murine undifferentiated neural progenitor cells.

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1. Introduction

Theanine (=γ-glutamylethylamide) is an amino acid ingredient in green tea with structural analogy to glutamine (Gln), which is a precursor for amino acid neurotransmitters such as γ-aminobutyric and glutamic acids, in the brain [1]. Although an intracerebroventricular injection of theanine protected hippocampal CA1 pyramidal neurons from delayed neuronal death in brains of gerbils with bilateral forebrain global ischemia *in vivo* [2], theanine was a poor inhibitor of ligand binding to different ionotropic glutamate receptor subtypes with high neurotoxicity in rat cortical synaptic membranes *in vitro* [3]. Theanine inhibited $[^3H]$Gln incorporation without affecting $[^3H]$glutamate uptake in rat brain synaptosomes, while both $[^3H]$theanine and $[^3H]$Gln were similarly taken up in a sodium- and structure-dependent manner [4]. Theanine intake was shown to promote the object recognition memory, along with an increase in 5-bromo-2′-deoxyuridine (BrdU) incorporation into the hippocampus, in developing young rats [5]. Similarly, theanine prevented the cognitive dysfunction in accelerated-senescence mice *in vivo* [6]. We have recently shown that daily oral intake of theanine alleviated a variety of behavioral abnormalities, in addition to preventing a transient decline of BrdU incorporation into the hippocampal dentate gyrus, in adult mice with traumatic severe stress [7].

In contrast, intracellular Gln was shown to participate in activation of the mammalian target of rapamycin (mTOR) kinase signaling system responsible for protein translation, cell growth and autophagy through a mechanism relevant to several membrane transporters for Gln in cultured HeLa cells [8]. Evidence for the

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mTOR pathway as an intracellular downstream signal of extracellular essential amino acids is now accumulating [9]. Marked promotion of cellular proliferation and neuronal differentiation was seen in pluripotent P19 cells with stable overexpression of solute carrier 38a1 (SLC38A1 = glutamine transporter, GlnT), which is responsible for the membrane transport of Gln in the brain [10]. Theanine similarly accelerated both activities in control stable transfectants with *empty vector* (EV), but failed to further facilitate the promotion of both proliferation and neuronal differentiation activities in stable *Slc38a1* transfectants [11].

These previous findings prompted us to investigate the activity of mTOR signaling-related molecules in neural progenitor cells exposed to theanine. For this purpose, we employed in this study neural progenitor cells isolated from embryonic mouse neocortex enriched of primitive cells immunoreactive for the undifferentiated progenitor cell marker nestin [12], in addition to the murine embryonal carcinoma cell line P19 cells with pluripotency. We also evaluated pharmacological actions of theanine on the proliferation in hippocampal progenitor cells [13] isolated from adult mice with predominant overexpression of green fluorescent protein (GFP) in cells expressing nestin [14].

2. Methods

2.1. Materials

Materials used were obtained from different sources described below. Theanine, Tokyo Kasei Kogyo (Tokyo, Japan); Pluripotent P19 stem cells derived from murine embryonal carcinoma, Riken Cell Bank (Tsukuba, Japan); Antibodies against microtubules-associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), ciliary neurotrophic factor (CNTF) and all-*trans* retinoic acid (ATRA), Sigma Chemicals (St. Louis, MO, USA); Antibody against BrdU, Abcam (Cambridge, UK); Antibodies for phosphorylated mTOR, p70 S6 kinase (p70S6K), phosphorylated-p70S6K and phosphorylated-S6, Cell Signaling Technology (Danvers, MA, USA); Anti-rabbit IgG antibody conjugated with peroxidase and ECL™ detection reagent, Amersham (Buckinghamshire, UK); Dulbecco's modified Eagle medium (DMEM), DMEM: Nutrient Mixture F-12 (DMEM/F-12) 1:1 Mixture, alpha minimal essential medium (αMEM), StemPro Accutase, GlutaMAX, B27 supplement and fetal bovine serum (FBS), GIBCO BRL (Gaithersburg, MD, USA), Epidermal growth factor (EGF) and fibroblast growth factor (FGF), Biomedical Technologies (Stoughton, MA, USA).

2.2. Animals use

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University (Permit no. 70093). All efforts were invariably made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques.

2.3. Embryonic mouse neural progenitor cells

Neocortex was isolated from 15.5-day-old embryonic Std-ddY mice (Japan SLC, Inc., Shizuoka, Japan), followed by trituration through a Pasteur pipette with enzyme cocktails in phosphate-buffered saline (PBS) and subsequent collection of the lower layer enriched of undifferentiated progenitor cells after Percoll centrifugation procedures [13,15]. Cells were cultured for 12 days under floating conditions at 37 °C under 5% CO_2 in a humidified CO_2 incubator with a half medium change every 2 days.

2.4. Proliferation

Cultured neural progenitor cells were exposed to theanine for 12 days in the presence of EGF. Proliferation activity was then quantified by MTT reduction assays [10]. Neurospheres cultured with theanine for 8 days were incubated with 10 μM BrdU for 10 h, followed by culture for 2 h and subsequent fixation with 4% paraformaldehyde (PA) for the immunocytochemical detection of BrdU [12]. Neurospheres were cultured with theanine for 6 days, followed by further culture for 2 h and fixation with 4% PA for double staining for DNA with 10 μg/ml Hoechst33342 and 5 μg/ml propidium iodide (PI) [12].

2.5. Differentiation

The cells were dissociated by pipetting, followed by seeding at 15,000 cells/well and subsequent culture without theanine for an additional 6 days [10]. Cells were then subjected to immunocytochemistry with antibodies against MAP2 (1:500) and GFAP (1:500) overnight at 4 °C. Quantification was performed by counting the number of cells immunoreactive for either MAP2 or GFAP in a blinded fashion, followed by calculation of the individual percentages over the number of total cells stained with Hoechst33342 [12]. For Western blotting, cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis [11] using antibodies against phospho-p70 S6 kinase (1:1,000), p70 S6 kinase (1:1,000), phosphor-S6 (1:1,000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:4,000). The relative amount of each protein was normalized by the quantitative densitometric analysis using Image J software.

2.6. Stable Slc38a1 transfectants

Mouse embryonal carcinoma P19 cells were plated at a density of 1.5×10^5 cells/cm^2, followed by culture in DMEM with 10% FBS for 24 h and subsequent stable transfection with pSI- GlnT, pSI-GFP and pcDNA3.1 vectors, or pSI, pSI-GFP and pcDNA3.1 vectors, using 2 μg of DNA and Lipofectamine and Plus reagent in 0.5 ml of Opti-MEM. Cells were selected after culture in medium containing 500 μg/mL of G418 for establishment of stable transfectants with *Slc38a1* expression vector and *EV* in P19 cells for further studies using clones between passages 3–6 as described previously [10].

2.7. Adult mouse neural progenitors

Hippocampus was isolated from 4-week-old *Nestin*-GFP transgenic mice, which were kindly provided by Dr. Grigori Enikolopov (Cold Spring Harbor Laboratory, NY, USA) [14], followed by mechanical trituration and culture in DMEM/F12 containing 1xGlutaMAX, 1xB27 supplement, 20 ng/mL EGF and 20 ng/mL FGF for 14 days under floating conditions. Resultant neurospheres were dissociated by StemPro Accutase, followed by culture at 300 cells/well with theanine in DMEM/F12 containing 1xGlutaMAX, 1xB27 supplement, 20 ng/mL EGF and 20 ng/mL FGF for an additional 24 days under floating conditions for the analysis of neurosphere size. Five different visual fields were chosen at random from each culture well under a phase contrast micrograph in a blinded fashion, followed by calculation of the size of neurospheres composed of clustered proliferating cells in parallel experiments for summation using the Scion Image β 4.02 software as described previously [12].

2.8. Data analysis

All results are expressed as the mean ± SE, and the statistical significance was determined by the one-way or two-way ANOVA

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