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NEUROPROTECTIVE EFFECTS OF YOKUKANSAN, A TRADITIONAL JAPANESE MEDICINE, ON GLUTAMATE-MEDIATED EXCITOTOXICITY IN CULTURED CELLS

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Abstract—To clarify the mechanism of yokukansan (TJ-54), a traditional Japanese medicine, against glutamate-mediated excitotoxicity, the effects of TJ-54 on glutamate uptake function were first examined using cultured rat cortical astrocytes. Under thiamine-deficient conditions, the uptake of glutamate into astrocytes, and the levels of proteins and mRNA expressions of glutamate aspartate transporter of astrocytes significantly decreased. These decreases were ameliorated in a dose-dependent manner by treatment with TJ-54 (100–700 μg/ml). The improvement of glutamate uptake with TJ-54 was completely blocked by the glutamate transporter inhibitor DL-threo-β-hydroxyaspartic acid. Effects of TJ-54 on glutamate-induced neuronal death were next examined by using cultured PC12 cells as a model for neurons. Addition of 17.5 mM glutamate to the culture medium induced an approximately 50% cell death, as evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. TJ-54 (1–1000 μg/ml) inhibited the cell death in a dose-dependent manner. Furthermore, competitive binding assays to glutamate receptors showed that TJ-54 bound potently to *N*-methyl-D-aspartate receptors, in particular, to its glutamate and glycine recognition sites. These results suggest that TJ-54 may exert a neuroprotective effect against glutamate-induced excitotoxicity not only by amelioration of dysfunction of astrocytes but also by direct protection of neuronal cells.

Key words: astrocytes, GLAST, GLT-1, mRNAs, NMDA, PC12.

Yokukansan (TJ-54) is one of the traditional Japanese medicines called "Kampo" medicine in Japan. It is composed of seven kinds of dried medical herbs. TJ-54 has been approved by the Ministry of Health, Labor and Welfare of Japan as a remedy for neurosis, insomnia, and irritability in children. Recently, TJ-54 was reported to improve behavioral and psychological symptoms of dementia (BPSD) such as hallucinations, agitation, and aggressiveness in patients with Alzheimer's disease, dementia with Lewy bodies, and other forms of senile dementia (Iwasaki et al., 2005a,b). However, the mechanism underlying the effectiveness of the medicine is still unclear.

Cognitive dysfunction and BPSD are thought to be associated with neurofunctional and neuropathological abnormalities in the brain. In several animal models used for studies in the pathogenesis of and therapy for dementia, an increase in the extracellular levels of the excitatory amino acids such as glutamate in the brain has been demonstrated (Todd and Butterworth, 2001; Han et al., 2008; Harkany et al., 2000; Behrens et al., 2002). Glutamate is well-known to contribute not only to induction of excitation of post-synaptic neurons but also to excitotoxic neuronal death due to the intensity and duration of glutamate exposure (Choi, 1988; Cheung et al., 1998). Under physiological conditions, neurotransmission to postsynaptic receptors is terminated by its clearance from the synaptic cleft by transporter proteins located in neuronal and astroglial cells to prevent an overload of glutamate to neurons. In particular, astrocytes take an important role in the efficient removal of glutamate from the extracellular space via two glutamate transporters, glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1) (Kanai et al., 1993; Schlag et al., 1998). These findings suggest that a failure of the glutamatergic system is related to the appearance of cognitive dysfunction and BPSD.

Previous studies have demonstrated that BPSD-like behaviors such as anxiety, depression, muricide, attacking, and startle responses as well as impairment of learning and memory are observed in thiamine-deficient (TD) rats and mice (Nakagawasai et al., 2000; Murata et al., 2004). Collins (1967) and Robertson et al. (1968) have demonstrated that astrocytes are among the first cells to be affected by thiamine deficiency in advance of neuronal cell death. Symptomatic TD is associated with increased extracellular glutamate concentrations in focal regions of the brain (Hazell et al., 1993; Langlais and Zhang, 1993) and TD in cultured astrocytes led to a decrease in the uptake of glutamate (Hazell et al., 2003). Therefore, dysfunction of the astrocyte glutamate transporter may be a major contributing factor to the increase in extracellular glutamate concentration and resulting excitotoxicity. In addition, excessive extracellular glutamate levels are believed to evoke neuronal dysfunction and in turn, neuronal

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Abbreviations: ANOVA, analysis of variance; BPSD, behavioral and psychological symptoms of dementia; cpm, counts per minute; DHK, dihydrokainate; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GFAP, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; GSH, glutathione; IC_{50}, half-maximal inhibitory concentration; mGluR, metabotropic glutamate receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; PBS(–), Ca-and Mg-free phosphate-buffered saline; PCP, phencyclidine; PKC, protein kinase C; RT, reverse transcription; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TBHA, DL-threo-β-hydroxy-aspartic acid; TD, thiamine deficiency; TJ-54, yokukansan.

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death, by excessive activation of *N*-methyl-D-aspartate (NMDA) receptors that leads to an intracellular cascade of cytotoxic events (Weikert et al., 1997).

Recently, we also confirmed that an increase in the extracellular level of glutamate and degeneration of neuronal and astroglial cells were observed in the brain of TD rats, and found that TJ-54 inhibited not only the TD-induced increase in extracellular level of glutamate but also the degeneration of cerebral neurocytes and astrocytes in the vulnerable brain regions (Kanno et al., 2008). Recently, *Uncaria rhynchophylla*, a constituent herb of TJ-54, was shown to protect against NMDA-induced neuronal excitotoxicity in rat hippocampus (Lee et al., 2003) or to block NMDA-induced current in cortical neurons (Sun et al., 2003). These results suggest that one of the mechanisms underlying the effects of TJ-54 may be closely related to improvement of glutamate excitatory neurotoxicity, including glutamate transporters and NMDA receptors as causative factors.

To clarify this hypothesis, we investigated the effects of TJ-54 on glutamate-mediated excitotoxicity by examining the effects on TD-induced decreases in glutamate uptake and glutamate transporters in cultured astrocytes, glutamate-induced PC12 cell death, and competitive binding to glutamate receptors in the rat brain membrane.

EXPERIMENTAL PROCEDURES

Drugs and reagents

TJ-54 used in the present study was a dry powdered extract from a mixture of *Atractylodes lancea* rhizome (4.0 g, rhizome of *Atractylodes lancea* De Candolle), Hoelen (4.0 g, sclerotium of *Poria cocos* Wolf), *Cnidii rizoma* (3.0 g, rhizome of *Cnidium officinale* Makino), *Uncaria* thorn (3.0 g, thorn of *Uncaria rhynchophylla* Miquel), Japanese angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa), *Bupleurum* root (2.0 g, root of *Bupleurum falcatum* Linné), and *Glycyrrhiza* root (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher) that was supplied by Tsumura & Co. (Tokyo, Japan).

Reagents used in cell culture experiments, DL-threo-β-hydroxy-aspartic acid (TBHA), dehydrokainate (DHK), pyrithiamine hydrobromide, Dulbecco's modified Eagle's medium (DMEM), DNase, glutamate dehydrogenase, β-nicotinamide adenine dinucleotide, 1-methoxyphenazine methosulfate, and Triton X-100, were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDTA, Hepes, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Dojindo (Kumamoto, Japan). Purified rabbit polyclonal antiserum to glial fibrillary acidic protein (GFAP) was purchased from Chemicon (Temecula, CA, USA). Vectastain Elite ABC (rabbit IgG) and DAB substrate kits for peroxidase were purchased from Vector Laboratories (Burlingame, CA, USA). Other chemicals were purchased from commercial sources.

For the Western blot analysis, a protease inhibitor cocktail, blocking buffer (SuperBlock), and Can Get Signal Solution were purchased from Sigma-Aldrich, Thermo Fisher Scientific (Rockford, IL, USA) and Toyobo (Osaka, Japan), respectively. Rabbit anti-GLT-1 and -GLAST were purchased from Wako (Osaka, Japan). Goat antiactin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-goat and sheep anti-rabbit horseradish peroxidase (HRP)–conjugated secondary antibodies were purchased from Sigma-Aldrich and GE Healthcare (Buckinghamshire, UK), respectively. ECL plus Western blotting detection regents were also obtained from GE Healthcare. Protein assay kit using Lowry method was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

For the real time reverse-transcription polymerase chain reaction (RT-PCR) assay, a Qiagen RNeasy mini kit was purchased from Qiagen (Hilden, Germany), and a TaqMan Gold RT-PCR Kit, TaqMan Universal PCR Master Mix, and TaqMan probes for detection of GLT-1, GLAST, and Rps29 were purchased from Applied Biosystems (Foster City, CA, USA).

Reagents used in the receptor binding assay, [^{3}H]CGP-39653 (NET-1050, specific radioactivity: 50 Ci/mmol), [^{3}H]TCP (NET-886, specific radioactivity: 41 Ci/mmol), and [^{3}H]ifenprodil (NET-1089, specific radioactivity: 43 Ci/mmol), were purchased from PerkinElmer (Waltham, MA, USA). [^{3}H]MDL-105519 (TRK-1043, specific radioactivity: 71 Ci/mmol) was purchased from GE Healthcare. Tris(hydroxymethyl)amino-methane (Tris), L-glutamic acid, ifenprodil, dizolcipin; (+)-MK-801, MDL-105519, 4-(2-hydroxyethyl)piperazine-(1) ethanesulfonic acid *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) and Hepes, were purchased from Sigma-Aldrich.

Preparation of primary astrocyte culture

Astrocytes were cultured by a modification of a procedure described previously (Juurlink and Walz, 1998). In brief, newborn rat neopallia were mechanically disrupted by pipetting in DMEM/Ca- and Mg-free phosphate-buffered saline [PBS(−)] (1:1). The suspension was filtered through a sterile nylon mesh with 100 μm pores (BD Falcon, Bedford, MA, USA). The filtrate was passed through a sterile lens cleaning paper (Fujifilm, Tokyo, Japan). Cells (3.75×10^6) were seeded into a 75 cm^2 culture flask (Corning, Corning, NY, USA) with DMEM containing 7.5 mM glucose, 2 mM glutamine, 25 mM $NaNCO_3$, and 10% horse serum. The next day, the culture medium was replaced with DMEM containing 25 mM sorbitol, 2 mM glutamine, 25 mM $NaNCO_3$, and 10% dialyzed horse serum, and the cells were incubated at 37 °C and 95% relative humidity provided with mixture of 5% CO_2 and 95% air for 2 weeks. After the incubation period, the cultures were returned to the glucose-containing DMEM medium, and the purity of astrocytes in the culture was examined by an immunocytochemical stain using an antibody to GFAP, which is a specific marker for astrocytes. We confirmed that at least 95% of cells were astrocytes.

The high purity–cultured astrocytes were removed from the substratum with Puck's solution, pH 7.2, containing 3 mM EDTA, 2 mM pyruvate, 7.5 mM glucose, 0.02% DNase and 10 mM Hepes. Approximately 20,000 cells per cm^2 were seeded into 96-well primaria culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) and used in the following experiments after the cells became confluent.

Examination of effects of TJ-54 on glutamate uptake in cultured astrocytes

TD astrocytes were prepared according to the procedure described by Hazell et al. (2003) to induce TD: the confluent astrocytes were transferred to 96 well-plate and cultured for 7 days in a custom-designed DMEM medium lacking in thiamine (CSTI, Sendai, Japan) and containing 5% horse serum in the presence of 10 μM pyrithiamine, which inhibits the enzyme responsible for production of the active form of thiamine. Control astrocytes were cultured in DMEM medium including thiamine and containing 5% horse serum for the same period. TD astrocytes were treated with TJ-54 as follows: astrocytes were cultured for 7 days in the TD medium with various concentrations of TJ-54 (final concentration: 100, 300, 500, or 700 μg/ml), which was filtered through a 0.22 μm filter.

Glutamate uptake ability was evaluated in cells cultured for 7 days. Namely, a 100 μM glutamate was exogenously added to astrocyte cultures. The aliquot of the culture medium was carefully