L-THEANINE PROTECTS THE APP (SWEDISH MUTATION) TRANSGENIC SH-SY5Y CELL AGAINST GLUTAMATE-INDUCED EXCITOTOXICITY VIA INHIBITION OF THE NMDA RECEPTOR PATHWAY

X. DI,[a,b] J. YAN,[b] Y. ZHAO,[c] J. ZHANG,[b] Z. SHI,[a]
Y. CHANG[a]* AND B. ZHAO[b]*

[a]*Institute of Molecular Neurobiology, Hebei Normal University, Shijiazhuang, 050016, PR China*

[b]*State Key Laboratory of Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Science, Beijing, 100101, PR China*

[c]*Department of Food Sciences and Technology, Harbin Institute of Technology, Weihai, 264209, PR China*

Abstract—As a natural analogue of glutamate, L-theanine is the unique amino acid derivative in green tea. Although its underlining mechanisms are not yet clear, it has been suggested that L-theanine treatment may prove beneficial to patients with neurodegenerative diseases. In this study, we investigated the neuroprotective effect and its mechanism of L-theanine in an *in vitro* model of Alzheimer's disease by using the human APP (Swedish mutation) transgenic SH-SY5Y cell. Amyloid beta (Aβ) neurotoxicity was triggered by L-glutamate in this cell line. Additionally, L-theanine significantly attenuated L-glutamate-induced apoptosis at similar levels to those seen with the NMDA receptor inhibitor MK-801 in the stably expressing APP Swedish mutation SH-SY5Y cells which over-generated Aβ. Meanwhile, the activation of c-Jun N-terminal kinase and caspase-3 induced by L-glutamate was suppressed by L-theanine. We also found that cells treated with L-theanine showed decreased production of nitric oxide resulting from the down-regulated protein levels of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS). These results indicate that the inhibition of the NMDA subtype of glutamate receptors and its related pathways is the crucial point of the neuroprotective effect of L-theanine in the cell model. Thus, our present study supports the notion that L-theanine may provide effective prophylaxis and treatment for Alzheimer's disease. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Alzheimer's disease, amyloid beta, L-theanine, calcium, nitric oxide, neuroprotection.

*Corresponding author. Tel: +86-106-4888569 or +86-311-86267215; fax: +86-106-4871293 or +86-311-87881815.
E-mail addresses: zhaobl@sun5.ibp.ac.cn (B. Zhao) or frankyzchang@yahoo.com.hk (Y. Chang).

Alzheimer's disease (AD) is an age-related neurodegenerative disorder, which is characterized by cognitive deterioration and the accumulation of amyloid beta (Aβ) peptides in specific brain regions (McKeel et al., 2004; Selkoe, 2002; Terry et al., 1991). Although multiple drugs including the noncompetitive inhibitor of the NMDA receptor memantine have now been approved for the treatment, their modest benefits and numerous side effects are discouraging. Mounting data indicate that Aβ peptides and the excitotoxicity induced by the NMDA receptor play the most important role in AD pathophysiology (Cotman and Su, 1996; Cotman et al., 1994; Selkoe, 2001; Stadelmann et al., 1999; Yankner, 1996). The latest studies describe a neurotoxic cycle in which the over-stimulation of NMDA receptors can accelerate Aβ production. This may subsequently induce excessive glutamate release and neuronal death (Lee et al., 2005; Lesne et al., 2005; Snyder et al., 2005; Townsend et al., 2007; Kodeeswaran et al., 2008). Glutamate toxicity, which results from over-activation of NMDA receptors and the subsequent increased amounts of intracellular calcium and nitric oxide, may trigger Aβ-induced cell death (Hynd et al., 2004). The stress-activated protein kinases, such as c-Jun N-terminal kinase (JNK), have been shown to be one of the signaling pathways activated by glutamate (Arthur et al., 2007; Chi et al., 2005; Yu et al., 2005). Many studies have reported that the stimulation of JNK in human and mouse AD brains is associated with the neuronal degeneration (Bozyczko-Coyne, 2002). JNK activation leads to activation of caspase-3, and expression of pro-apoptotic genes (Culmsee and Mattson, 2005). Therefore, the inhibition of these pathways could prove beneficial in the treatment of AD.

L-theanine is the major amino acid component in green tea; and it is also widely used as a food additive (Finger et al., 1992). As a natural antagonist of glutamate, L-theanine can inhibit the re-uptake of glutamate from the synaptic cleft and block the glutamate receptors in the hippocampus (Kakuda et al., 2002). Meanwhile, recent studies suggest that L-theanine is a cognitive performance enhancer (Haskell et al., 2005). According to these studies, we assumed that L-theanine may protect neurocyte against apoptosis induced by L-glutamate and Aβ. In this study, we established an *in vitro* model of AD by using L-glutamate to induce excitotoxicity in the human APP Swedish mutation (APPsw) transgenic SH-SY5Y cells. Based on this model, we found that the SH-SY5Y cell, which stably expresses APPsw, was very sensitive to L-glutamate-induced excito-

toxicity. To verify our hypothesis, we identified the effects of L-theanine and glutamate on amyloid peptide Aβ$_{1-40}$ secretion in APPsw cells, and detected whether L-theanine can ameliorate the decrease in observed glutamate-induced cell viability. We further used the NOS inhibitor L-NMMA and the NMDA receptor inhibitor MK-801 to demonstrate the essential roles of nitric oxide (NO) and the NMDA receptor in L-glutamate-induced neurocyte apoptosis in APPsw cells, respectively. Then, we determined the downstream mechanism of NMDA receptor by measuring intracellular calcium concentrations and the NO levels. We also assessed the expressions of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS), as well as the apoptosis-signaling molecules phosphorylated-c-Jun N-terminal kinase (p-JNK) and caspase-3, in order to dissect out the neuroprotective mechanisms of L-theanine.

EXPERIMENTAL PROCEDURES

Reagents

Dulbecco's modified Eagle's medium (DMEM), Fetal calf serum, HEPES and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from GIBCO BRL (Grand Island, NY, USA). 3-Amino,4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA), DNase I, ethylenediaminetetraacetic acid (EDTA), Hoechst 33258, N^G-methy-L-arginine acetate salt (L-NMMA), MK-801, trypsin, penicillin, propidium iodide (PI), and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Fluo-3 Acetoxymethyl ester (Fluo-3 AM) was purchased from Calbiochem (San Diego, CA, USA); Antibodies of nNOS (sc-648), iNOS (sc-651), caspase-3 (sc-7148), p-JNK (sc-6254) and β-Actin (sc-1616-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human β Amyloid 1–40 Colorimetric Immunoassay Kit was purchased from Invitrogen Corporation (Invitrogen, Carlsbad, CA, USA). L-theanine, with the purity of 98.11% (analyzed by HPLC), is a generous gift from Hangzhou Gosun Biotechnologies Co., Ltd. (Hangzhou, PR China). All other chemicals made in China were analytical grade.

Cell culture, transfection and treatment

Human neuroblastoma SH-SY5Y cells were maintained in a medium consisting of DMEM supplemented with fetal calf serum (10% v/v), penicillin (100 IU/ml), and streptomycin (100 μg/mL) in humidified 5% CO_2/ 95% air at 37 °C to generate SH-SY5Y cell lines stably expressing human APP (wild type), APPsw (APP Swedish mutation) or empty vector (neo) pCLNCXv.2, cells were transfected using lipofectamine 2000 (Invitrogen Inc., USA) reagents and selected with G418. Cells were planted at a density of 2×10^5 cells/mL on 96-well plates and 2×10^6 cells/mL in 25 cm^2 flasks according to a previously described protocol (Zhang and Zhao, 2003). To investigate the excitotoxicity of L-glutamate, cells were treated with different concentrations of L-glutamate (0.1–10 mM) for 12 h to investigate the excitotoxicity of L-glutamate. L-theanine (0.1, 0.5, or 1 mM) was added to cells 2 h before and then incubated with 1 mM L-glutamate for 12 h. For glutamate exposure, the culture medium was replaced with Lock's buffer- (NaCl 154 mM, KCl 5.6 mM, NaHCO$_3$ 3.4 mM, MgCl$_2$ 1.2 mM, glucose 5.6 mM, HEPES 5 mM and CaCl$_2$ 2.3 mM, pH 7.4) (Grynkiewicz et al., 1985).

Assessment of cell viability

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bro-

mide), showing the mitochondrial activity of living cells as described in published literatures (Denizot and Lang, 1986; Mossmann, 1983). After different treatments, 500 μg/mL MTT (final concentration) was added to per well and than incubated at 37 °C for 3 h in 96-well plates. MTT was removed, and the crystals were dissolved by dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured using a Bio-Rad 3350 Microplate Reader. Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

Flow cytometric analysis using PI

Flow cytometric analysis was used to quantify apoptosis, cells (2×10^6 cells) were added to each flask to conduct the flow cytometry. After treatment, cells were centrifuged (1200 g, 5 min) and washed three times with phosphate-buffered saline (PBS) to remove the medium. After cells were fixed in 70% cold ethanol overnight, the fixed cells were washed twice and resuspended in 0.5 ml PBS (containing 50 μg/mL RNaseA), and incubated for 30 min at 37 °C, PI (50 μg/mL) was added, gently mixed and incubated for 30 min at room temperature (15–25 °C) in darkness. The samples were then detected in the FL2-H channels of an FACS Calibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA), using emission filters of 575 nm. Analyses were performed by the software supplied with the instrument.

Intracellular calcium concentration ([Ca^{2+}]$_i$)

The concentration of intracellular Ca^{2+} was measured with Fluo-3 AM by the method of Aoshima et al. and with some modifications (Aoshima et al., 1997). Briefly, after treatment, cells were harvested, washed and resuspended in a standard medium (containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 5.6 mM glucose, 1.5 mM CaCl$_2$, and 20 mM HEPES (final pH 7.4), Fluo-3 AM (5 μM)) was added and subsequently incubated for 30 min at 37 °C. After washing three times, cells were resuspended in the standard medium and transferred to a fluorometer cuvette. The fluorescence intensity of Fluo-3 was quantified by a fluorescence spectrophotometer (Hitachi F-4500) at an excitation wavelength of 490 nm and an emission wavelength of 526 nm. [Ca^{2+}]i was calculated from the Fluo-3 fluorescence intensity using the equation: [Ca^{2+}]$_i$=K$_d$ (F$_0$−F$_{min}$)÷(F$_{max}$−F$_0$)(nmol/l). For the purpose of calculation of [Ca^{2+}]i, the K$_d$ was assumed to remain constant between 10 and 25 °C, and increase linearly up to 42 °C and K$_d$=400 nmol/l at 25 °C. The maximal Fluo-3 fluorescence intensity (F$_{max}$) was determined by adding 0.1% Triton X-100 and the minimal fluorescence (F$_{min}$) was determined by quenching Fluo-3 fluorescence with 5 mM (−)-epigallocatechingallate (EGTA). F$_0$ is the fluorescence measured without Triton X-100 or EGTA.

Measurement of intracellular NO

The intracellular NO was detected using DAF-FM DA as described by Nakatsubo et al. with some modifications (Nakatsubo et al., 1998). DAF-FM DA, a nitric oxide fluorescent probe, can react with NO within variable cells to produce a fluorescent compound. The cells were collected and resuspended in BSS (containing 130 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, 15 mM glucose, and 5 mM HEPES, pH 7.4) and then incubated with 5 μM DAF-FM DA (in DMSO) for 45 min at 37 °C. After washing out the excess probe, the fluorescence intensity was recorded in the Hitachi F-4500 at 485 nm excitation and 515 nm emission.

Western blotting

The cells were collected and lysised on ice for 30 min by lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 100 μg/mL PMSF, 1 μg/mL aprotinin and 1% Triton X-100). The lysates were centrifuged at

12,000 g for 20 min at 4 °C and supernatants were collected. The protein content was estimated by BCA kit (Pierce Inc., USA) and the proteins were separated by sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE) and transferred to a ni-trocellulose membrane. The membrane was incubated in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 5% non-fat milk) overnight at 4 °C. After that, the membrane was incubated for 2 h with the antibody against iNOS, nNOS, caspase-3, p-JNK or Actin in 1:500 dilution; followed by incubating with peroxidase-conjugated secondary antibody for 1 h with con-stant agitation, then washed and reacted with the supersignal chemiluminescent substrate (Pierce Biotechnology, IL, USA), and exposed to Kodak-XAR film. The film was digitized, and the band intensities were analyzed by an NIH imaging software. The anti-body of iNOS is an affinity purified rabbit polyclonal antibody raised against a peptide mapping near the N-terminus of NOS2 of human origin, and it is recommended for detection of iNOS of mouse, rat, and human origin by Western blotting; antibody of nNOS is an affinity purified rabbit polyclonal antibody raised against a peptide mapping near the C-terminus of NOS1 of rat origin, and it is recommended for detection of NOS1 of mouse, rat and human origin by Western blotting; antibody of caspase-3 is a rabbit polyclonal antibody raised against amino acids 1-277 rep-resenting full length procaspase-3 of human origin, and it is rec-ommended for detection of caspase-3 p11, p17 and p20 subunits and full length procaspase-3 of mouse, rat and human origin by Western blotting; antibody of p-JNK is a mouse monoclonal anti-body raised against a sequence containing phosphorylated Thr 183 and Tyr 185 of JNK of human origin, and it is recommended for detection of JNK1, JNK2 and JNK3 phosphorylated at Thr 183 and Tyr 185 of mouse, rat and human origin by Western blotting; and the antibody of Actin is a rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of Actin of human origin, and it is recommended for detection of a broad range of Actin isoforms of mouse, rat, human, zebrafish, *C. elegans*, Dro-sophila, *S. cerevisiae* and *Xenopus* origin by Western blotting.

Conditioned medium preparation and Aβ detection by sandwich ELISA

After treatment, the supernatants of cell culture were harvested, centrifuged and 10×concentrated in presence of a protease in-hibitors (2.5 mM EDTA, 10 μM leupeptin, 1 μM peptastin, 1 mM phenylmethylsulfonyl fluoride) cocktail using Centricon (Amicon) with a cutoff value of 3 kDa. The conditioned medium superna-tants were stored at −70 °C for sandwich ELISA analysis. Aβ production was measured by a sensitive fluorescence based sandwich ELISA assay using a kit (Human β Amyloid 1−40 Col-orimetric Immunoassay Kit) from invitrogen Corporation according to the manufacturer's instructions.

Statistical analysis

All experiments were done in triplicate. One-way ANOVA was used to estimate overall significance followed by post hoc Tukey's tests corrected for multiple comparisons. Data are presented as mean±SEM. A probability level of 5% ($P<0.05$) was considered significant.

RESULTS

L-theanine ameliorated the decreases of cell viability induced by L-glutamate in the APPsw cell

There was no significant difference of cell viability among the neo, APP and APPsw cells without any treatment. After cells were treated with L-theanine, the cell viability was enhanced in a dose-dependent manner, and this effect

was saturated at a concentration of 0.5 mM (Fig. 1A). Therefore, 0.5 mM was selected as the working concen-tration of L-theanine in the following experiments. As pre-dicted, the cell viability was decreased by about 12%, 18%, and 21% in the presence of 100 μM L-glutamate in neo, APP, and APPsw cells, respectively. When the glutamate concentration was increased to 1 mM, cell viability was decreased by about 31%, 35%, and 57% in neo, APP, and APPsw cells, respectively (Fig. 1B). The decreases in cell viability induced by L-glutamate in neo and APP cells were not as acute as those in APPsw cells. This suggests that the expression of APPsw in SH-SY5Y cells rendered cells more susceptible to glutamate-induced excitotoxicity. Therefore, APPsw cells treated with glutamate were used as an in vitro model of AD for further experiments in this study. Pretreatment of cells with L-theanine for 2 h pro-tected neo (Fig. 1C) and APPsw (Fig. 1D) cells from L-glutamate-induced cell toxicity in a dose-dependent man-ner (0.1–1 mM). Due to the fact that excessive activation of the NMDA receptor and overproduction of NO play impor-tant roles in glutamate-induced cytotoxicity, we also tested the influence of the inhibitors of the NMDA receptor (MK-801) or NOS (L-NMMA) on the decreases in cell viability induced by L-glutamate in APPsw cells. As shown in Fig. 1E, F, MK-801 (NMDA receptor inhibitor) and L-NMMA (NOS inhibitor) pretreatment attenuated the decreases of cell viability induced by L-glutamate.

L-theanine attenuated the apoptosis induced by L-glutamate

The apoptosis of APPsw cells was determined using flow cytometry method. After treated with 1 mM of L-glutamate, it was found that the apoptotic rate was about 30% in cells (Fig. 2B, G). As shown in Fig. 2D–F, L-theanine, MK-801, or L-NMMA pretreatment protected cells from L-glutamate-induced apoptosis, while L-theanine alone did not exhibit any significant effect on the cell's activity (Fig. 2C). Both the inhibitor of the NMDA receptor and the inhibitor of NOS could attenuate apoptosis of APPsw cells induced by L-glutamate. This suggests that excessive activation of the NMDA receptor and overproduction of NO contributed to the glutamate-induced apoptosis. As a natural antagonist of glutamate, L-theanine ameliorated glutamate-induced apoptosis in a way similar to that of the NMDA receptor inhibitor and the inhibitor of NOS, indicating that it may protect APPsw cells from glutamate-induced apoptosis via inhibition of NMDA receptor over-activation, NO overpro-duction, and their related pathways.

L-theanine inhibited L-glutamate induced elevation of [Ca^{2+}]$_i$

Since the disturbance of Ca^{2+} homeostasis is involved in the neuronal death present in AD (Hynd et al., 2004), and because L-glutamate can increase intracellular calcium levels via activation of the NMDA receptor, we examined the intracellular calcium concentration with the Fluo-3 AM fluorescence probe. As shown in Fig. 3; 1 mM L-glutamate treatment increased the calcium level nearly twofold in APPsw cells and 140% in neo cells, while 0.5 mM L-

Fig. 1. Effects of L-glutamate and L-theanine on the neo, APP and APPsw cell viability. Panel (A, B) show the effects of different concentrations of L-theanine and L-glutamate on the cell viability in neo, APP and APPsw cells. In panel (A, B), * $P<0.05$; # $P<0.05$; $ $P<0.05$ compared with L-glutamate (0) treatment in neo, APP and APPsw cells. Panel (C, D) show the effects of L-glutamate and L-theanine on the neo and APPsw cell viability respectively. In panel (C, D), * $P<0.05$ compared with (L-glutamate 0; L-theanine 0); # $P<0.05$ compared with (L-glutamate 1; L-theanine 0). Panel (E, F) show the effect of MK-801 (the inhibitor of NMDA receptor) and L-NMMA (the inhibitor of NOS) on the cell viability decreased by L-glutamate. * $P<0.05$ compared with (L-glutamate 0; L-theanine 0); # $P<0.05$ compared with (L-glutamate 1; L-theanine 0). The results were expressed in mean±SEM, $n=6$. The cells were treated by different compounds and the cell viability was measured by quantitative colorimetric assay with MTT. The details were described in the methods.

theanine pretreatment significantly reversed this elevation of the calcium level. These results indicate that the increase of intracellular $[Ca^{2+}]_i$ levels were higher in APPsw cells than in neo cells. Furthermore, L-theanine prevented the disturbance of Ca^{2+} homeostasis induced by glutamate.

Effects of L-theanine on the intracellular NO level and the expressions of nNOS and iNOS

Recent evidence suggests that NO may play an important role in AD and other neurodegenerative disorders. A previous study demonstrated that stimulation of the NMDA receptor can result in excessive NO formation (Hynd et al., 2004). Therefore, in the present study we examined the effect of L-theanine on intracellular NO production and the protein expressions of iNOS and nNOS. As shown in Fig.

4, L-theanine treatment alone decreased the intracellular NO content by about 17% in APPsw cells. L-glutamate administration significantly increased the generation of NO, while pretreatment with L-theanine decreased this elevation of NO by about 40%. As shown in Fig. 5, 1 mM of L-glutamate treatment increased the expressions of nNOS and iNOS by about 45% and 56%, respectively, while L-theanine pretreatment blunted this up-regulation. These results suggest that L-theanine inhibited the NO overproduction induced by glutamate.

L-theanine attenuated the up-regulation of p-JNK and caspase-3 induced by L-glutamate

JNK phosphorylation has been identified as one of the signaling pathways which can be activated by glutamate,
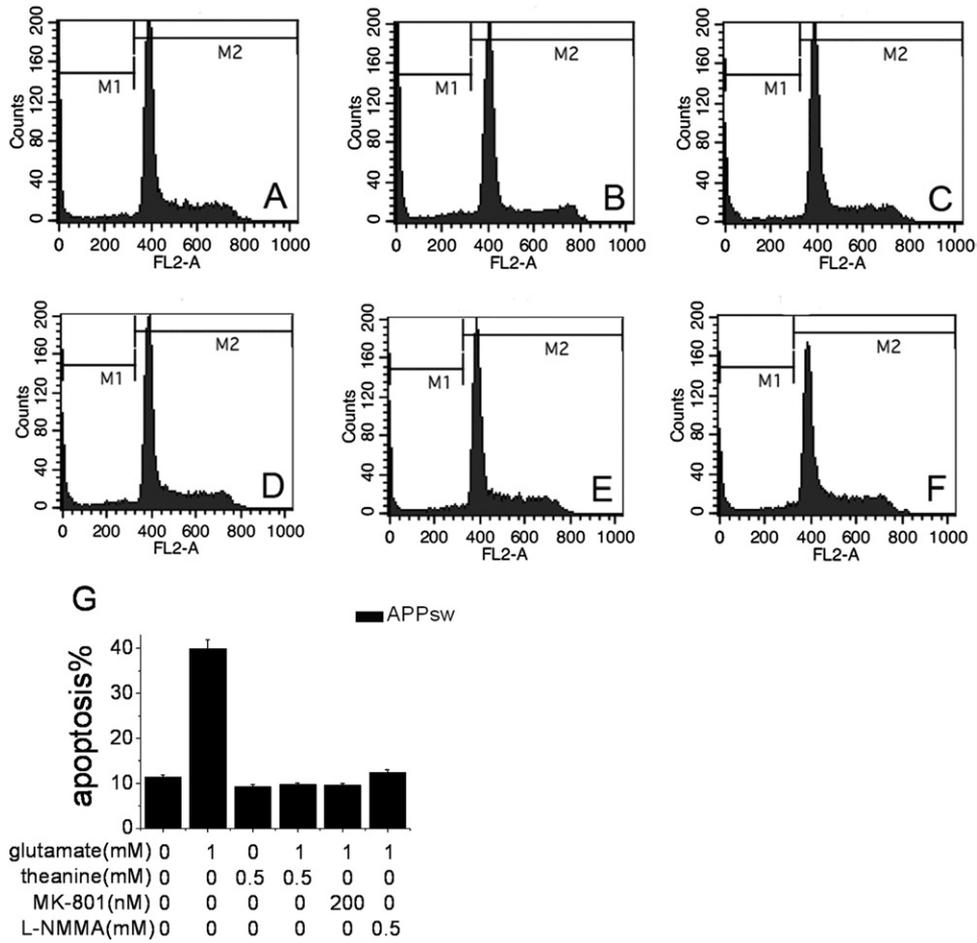
Fig. 2. Effects of L-glutamate, L-theanine, MK801 (the inhibitor of NMDA receptor) and L-NMMA (the inhibitor of iNOS) on apoptosis ratio in APPsw cells. Untreated cells (A), L-glutamate (1 mM) treated (B), L-theanine (0.5 mM) treated (C), cells pre-treated with L-theanine (0.5 mM) (D) or MK-801 (200 nM) (E) or L-NMMA (0.5 mM) (F), then exposed to L-glutamate. The mean±SEM for three independent experiments (G). The cells were treated with different compounds and examined by flow cytometry. The details were described in the methods.

and the up-regulation of caspase-3 is an important apoptosis marker (Culmsee and Mattson, 2005; Arthur et al., 2007). Therefore, we examined the effect of L-theanine on JNK activation and caspase-3 protein expression in
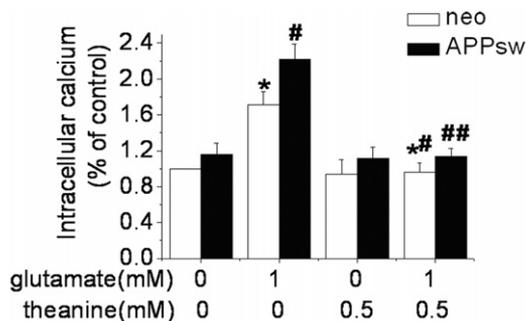
Fig. 3. Intracellular calcium levels in neo and APPsw cells were detected by Fluo-3 fluoresce. The intracellular $[Ca^{2+}]_i$ was measured after neo or APPsw exposed to L-glutamate or L-theanine with probe Fluo-3 AM. The result is expressed in mean±SEM, $n=4$. * $P<0.05$ compared with (neo L-glutamate 0; L-theanine 0); # $P<0.05$ compared with (APPsw L-glutamate 0; L-theanine 0); *# $P<0.05$ compared with (neo L-glutamate 1; L-theanine 0); ## $P<0.05$ compared with (APPsw L-glutamate 1; L-theanine 0). The details were described in the methods.
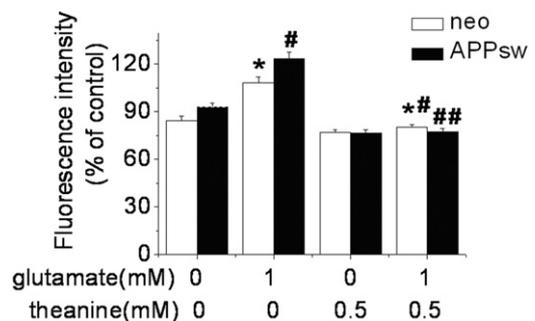
Fig. 4. The relative content of nitric oxide generated in the cells treated by L-glutamate or L-theanine. Nitric oxide was measured using DAF-FM DA as a probe to detect its fluorescence intensity. The result is expressed in mean±SEM of a ratio between fluorescence intensity and protein content, $n=4$. * $P<0.05$ compared with (neo L-glutamate 0; L-theanine 0); # $P<0.05$ compared with (APPsw L-glutamate 0; L-theanine 0); *# $P<0.05$ compared with (neo L-glutamate 1; L-theanine 0); ## $P<0.05$ compared with (APPsw L-glutamate 1; L-theanine 0). The details were described in the methods.
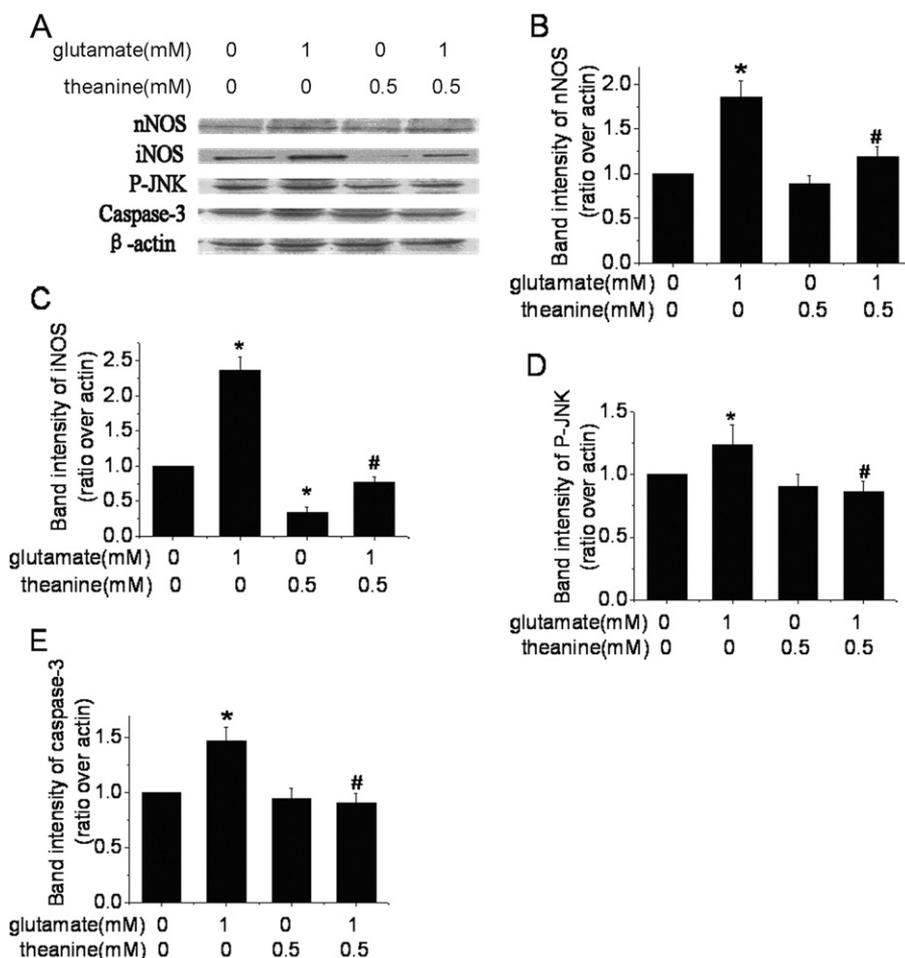
Fig. 5. Effects of L-glutamate and L-theanine on the expression of nNOS, iNOS, P-JNK and caspase-3 in the APPsw cells measured by western blot. nNOS, iNOS, p-JNK and caspase-3 expressions in the APPsw cells after different treatment (A). The statistic results of band intensities were shown below: nNOS (B), iNOS (C), p-JNK (D), and caspase-3 (E). The data were expressed as ratio±SEM, $n=5$. * $P<0.05$ compared with (L-glutamate 0; L-theanine 0); $^\#$ $P<0.05$ compared with (L-glutamate 1; L-theanine 0). The detail were described in the methods.

APPsw cells. Fig. 5D, E shows that after the L-glutamate treatment, expressions of p-JNK and caspase-3 were increased about 30% and 48%, respectively. Following L-theanine pretreatment, however, these up-regulations of protein levels induced by L-glutamate decreased about 40% and 50%, respectively.

**L-theanine decreased the amount of Aβ peptide secreted to the medium**

Aβ is a 39–43 amino acid peptide produced from APP, and is the major constituent of the amyloid core of senile plaque. $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most predominant species found in AD brains (Hsieh et al., 2006). $A\beta_{1-40}$ alone accounts for about 90% of secreted Aβ peptide (Citron et al., 1996). The level of $A\beta_{1-40}$ increases proportionately more in AD and may correlate better with synaptic change and cognitive deficits (Lue et al., 1999). Snyder and his team (Snyder et al., 1994) found that $A\beta_{1-42}$ aggregates more readily than the other molecules. In the present study, we quantified the content of $A\beta_{1-40}$ in culture medium of APPsw cells using a sandwich ELISA kit to eval-

uate the role of L-theanine on the Aβ peptide secretion. As shown in Fig. 6, L-theanine treatment alone decreased the secretion of Aβ peptide by 18%, whereas 1 mM of L-glutamate administration increased the $A\beta_{1-40}$ secretion by 30%. After L-theanine pre-administration, this elevation of Aβ peptide secretion was significantly decreased.

DISCUSSION

The APPsw cells were susceptible to glutamate-induced excitotoxicity

The stable expression of a mutant APP gene in neuronal cell lines to generate Aβ has been used as a classical method to generate the AD model in vitro. Previously, we found that the APP protein was over-generated in both APP cells (SH-SY5Y cell lines stably over-expressed human wide-type APP) and APPsw cells (SH-SY5Y cell lines stably expressed APP Swedish mutation); yet Aβ was only over-generated in APPsw cells (Zhang et al., 2006). Our present results agree with previous reports that the APP Swedish mutation could induce the over-generation of Aβ.
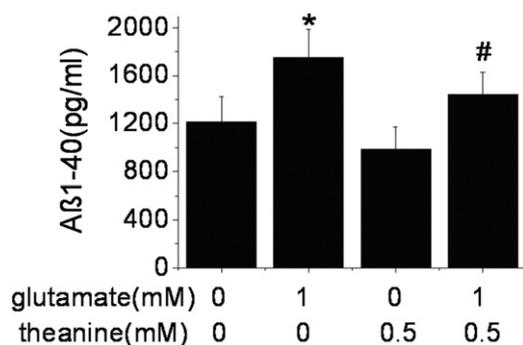
Fig. 6. Contents of $A\beta_{1-40}$ in the conditioned media were quantified by ELISA. The effect of L-glutamate and L-theanine on $A\beta$ secretion of the APPsw cells was measured by a sensitive fluorescence-based sandwich ELISA assay using a kit (The Human β Amyloid 1−40 colorimetirc Immunoassay Kit). The result is expressed in mean±SEM, $n=4$. * $P<0.05$ compared with (L-glutamate 0; L-theanine 0); # $P<0.05$ compared with (L-glutamate 1; L-theanine 0). The detail were described in the methods.

In the normal growth environment, the over-generated $A\beta$ did not demonstrate any toxicity to the cell (Fig. 1). After administration of L-glutamate in the medium, the cell viability was decreased by about 31% in neo (SH-SY5Y cell lines stably transfected with empty vector pCLNCXv.2) cells, a 57% decrease in APPsw cells (Fig. 1B). A further study found that L-glutamate administration increased the $A\beta$1-40 secretion about 30% in APPsw cells (Fig. 6). This result may be achieved by inhibiting α-secretase activity, which resulted from over-stimulation of NMDA receptors (Lesne et al., 2005). Therefore, we used APPsw cells treated with L-glutamate as an in vitro model of AD. In this model, L-glutamate and $A\beta$ interact to form a neurotoxic feedback cycle (Kodeeswaran et al., 2008). Evidence from our study indirectly demonstrates that the synergistic effect of neurotoxicity is induced by L-glutamate and the over-expression of APP Swedish mutation ($A\beta$ overproduction). In our present study, we successfully used the SH-SY5Y cell lines to generate an in vitro model of AD; however, the susceptibility of L-glutamate and $A\beta$-induced toxicity in other cell lines still need to be demonstrated in further studies.

L-theanine ameliorated the decreases of cell viability induced by L-glutamate at similar levels as seen with MK-801 and L-NMMA in the APPsw cell

Previous reports have shown that green tea components such as L-theanine have neuroprotective properties; however, a clear cellular mechanism underlying these effects has not yet been established (Egashira et al., 2004; Kakuda, 2002; Nathan et al., 2006). L-theanine is a glutamate analog that can bind to AMPA, kainite and NMDA receptors with low affinity. It may also produce neuroprotective effects upon binding to these receptor subtypes (Kakuda et al., 2002). It has been reported that both ionotropic and metabotropic glutamate receptors expressed in SH-SY5Y cells, which may be useful in exploring excitatory amino acid-induced processes in human neuronal tumor cells (Naarala et al., 1993; Nair et al., 1996). In our present

study, we found that L-theanine ameliorated the decreases of cell viability induced by L-glutamate at similar levels as seen with MK-801 and L-NMMA in the APPsw cells (Fig. 1D–F), which suggests that the decreases of cell viability induced by L-glutamate in APPsw cells are due at least partially to the excessive stimulation of the NMDA receptor and the over-production of NO. As a glutamate analog, L-theanine plays a role similar to MK801 or L-NMMA in inhibiting excitotoxicity, which agrees with the previous reports which state that glutamate triggers $A\beta$ toxicity-particularly that which is mediated by NMDA receptors (Lesne et al., 2005). Thus, L-theanine may exert neuroprotective effects at least partially through blunting NMDA receptor-related pathways.

L-theanine, MK-801, and L-NMMA attenuated the apoptosis in APPsw cells

L-glutamate-induced excitotoxic neuronal death via over-stimulation of the NMDA receptor associated with excessive Ca^{2+} influx. Even more important is that the abnormalities in Ca^{2+} homeostasis induced by the $A\beta$ peptide might be involved in the pathophysiology of AD (Liu and Zhao, 2004; Mattson, 1994; Zeng et al., 2004). The oxidative damage caused by L-glutamate, the $A\beta$ peptide, and the activation of Ca^{2+}-dependent enzymes may account for the disruption of Ca^{2+} homeostasis (Choi, 1992; Mattson, 1994). Over the course of this study, we found that the L-glutamate induced both a rise in calcium levels (Fig. 3) and apoptosis (Fig. 2) in APPsw cells, while L-theanine attenuated this rise in calcium concentration (Fig. 3). Moreover, pre-administration of the L-theanine or MK-801 in the cell culture medium suppressed the L-glutamate-induced cell apoptosis in our model (Fig. 2). These data indicate that the increase of the cellular calcium levels in APPsw cells may be the underlying mechanism of the neuronal toxicity. L-theanine, as the natural antagonist of glutamate, prevented the disruption of Ca^{2+} homeostasis. Several lines of evidence also suggest that NO may be directly related to neuronal death in AD (Dawson and Dawson, 1998; Kaufman, 1999; Luth and Arendt, 1998). Both the NMDA receptor activation and the $A\beta$ aggregation could cause the increases in the expression of iNOS and the production of NO, which subsequently lead to excessive damage caused by free radicals (Akama and Eldik, 2000; Keil et al., 2004). Our results indicate that the protein levels of iNOS and nNOS, and the production of NO increased significantly in APPsw cells treated with L-glutamate, while L-theanine down-regulates the NO production and protein levels of iNOS and nNOS (Figs. 4 and 5). Moreover, pretreatment of APPsw cells with L-theanine or the NOS inhibitor L-NMMA prevented apoptosis triggered by L-glutamate (Fig. 2). These data suggest that L-theanine could potentially protect the neurocyte from excitotoxicity probably through the inhibition of NO overproduction.

L-theanine modulates JNK-related cell signaling pathways

Evidence from published data show that JNK was involved in the neuronal cell death as a downstream regulator of the

NMDA receptor and Aβ (Arthur et al., 2007; Chi et al., 2005; Yu et al., 2005). Ca^{2+} accumulation could result in JNK phosphorylation through activation of Ca^{2+}-dependent enzymes. This would in turn activate caspase-3 and induce apoptosis (Chi et al., 2005). Taken as a whole, the evidence from our study suggests that L-glutamate and Aβ may induce cellular Ca^{2+} accumulation along with over-activation of the NMDA receptor in APPsw cells. The disruption of Ca^{2+} homeostasis may induce the phosphorylation of the JNK and over-production of NO. Our results show that L-glutamate up-regulated the phosphorylation of JNK and expressions of caspase-3, and the effects of L-glutamate were blocked by pretreatment with L-theanine in APPsw cells (Fig. 5). These findings indicate that L-theanine at least partially protected the APPsw cells from apoptosis induced by glutamate through the JNK signaling pathway.

Prospect of medical applications and safety of L-theanine

The safety of L-theanine is important when considering its potential applications. When L-theanine was administered as a dietary mixture to rats for 13 weeks, there were no adverse effects observed, even at a dosage of 4000 mg/kg/day (Borzelleca et al., 2006). The role of L-theanine in improving memory and learning performance in rats was reported at a dose of 180 mg/kg daily for 4 months (Juneja et al., 1999). In China, the average person drinks around 10 g of tea per day, while the "heavy" drinkers consume around 30 g/day. According to previous data, 30 g of tea contains 300 mg of L-theanine which equals 5 mg/kg/day (green tea contains about 1% L-theanine, and body weight is normalized as 60 kg) (Ekborg-Ott et al., 1997). Previous literature showed that 0.5–2 h were needed to reach the peak absorption of L-theanine after oral administration (Kakuda, 2002). L-theanine could pass through the blood–

brain barrier via the leucine-preferring transport system (Yokogoshi et al., 1998).

CONCLUSION

L-theanine can protect cells against L-glutamate-induced neurotoxicity in the APPsw over-expressed SH-SY5Y cell. This effect is achieved via the over-activation of the NMDA receptor and its related pathways. As shown in Fig. 7, L-theanine acts as a natural antagonist of glutamate. It prevents the increase of Aβ secretion induced by the over-activation of the NMDA receptor, modulates Ca^{2+} and NO-related cell-signaling pathways and protects the neurocyte from apoptosis. Thus, our present data support the notion that L-theanine may provide effective prophylaxis and treatment for Alzheimer's disease, but further in vivo investigations are needed.

Fig. 7. The model diagram of the mechanism of the protective effect of L-theanine on the glutamate induced cell apoptosis in the APPsw cell: (1) inhibition of NMDA subtype of glutamate receptors over activation and Aβ secretion; (2) preventing the disturbance of Ca^{2+} homeostasis; (3) inhibiting the overproduction of NO; and (4) modulating the level of phosphorylative of JNK and caspase-3.

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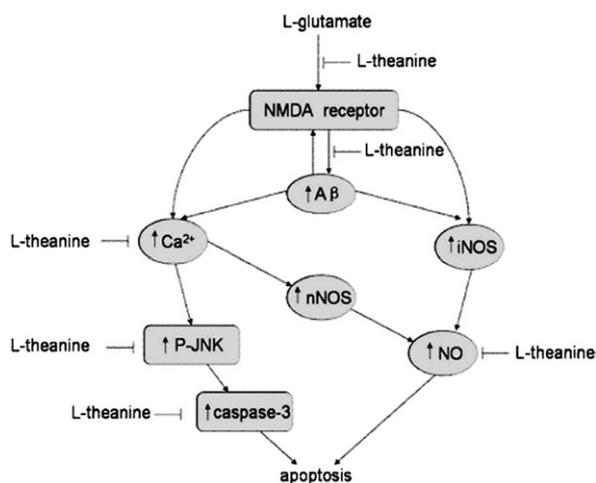
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