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Neuroprotective Effect of L-Theanine on Aβ-Induced Neurotoxicity through Anti-Oxidative Mechanisms in SK-N-SH and SK-N-MC Cells

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Abstract

Amyloid beta (Aβ)-induced neurotoxicity is a major pathological mechanism of Alzheimer's disease (AD). In this study, we investigated the inhibitory effect of L-theanine, a component of green tea (Camellia sinensis) on $A\beta_{1-42}$-induced neurotoxicity and oxidative damages of macromolecules. L-theanine inhibited $A\beta_{1-42}$-induced generation of reactive oxygen species, and activation of extracellular signal-regulated kinase and p38 mitogenic activated protein kinase as well as the activity of nuclear factor kappa-B. L-theanine also significantly reduced oxidative protein and lipid damage, and elevated glutathione level. Consistent with the reduced neurotoxic signals, L-theanine (10-50 μg/ml) concomitantly attenuated $A\beta_{1-42}$ (5 μM)-induced neurotoxicity in SK-N-MC and SK-N-SH human neuroblastoma cells. These data indicate that L-theanine on Aβ-induced neurotoxicity prevented oxidative damages of neuronal cells, and may be useful in the prevention and treatment of neurodegenerative disease like AD.

Key Words: L-theanine, Amyloid beta, Oxidative stress, Neuronal damages

INTRODUCTION

Extensive genetic and experimental evidence have shown that Aβ is the critical factor in AD pathogenesis (Selkoe, 2000; Lecanu *et al.*, 2004; Cerpa *et al.*, 2008). It is widely acknowledged that Aβ is associated with oxidative stress damage in the AD brain (Butterfield and Lauderback, 2002; Abdul *et al.*, 2008). The consequences of Aβ-associated oxidative stress are mitochondrial impairment, loss of synapses and neurons, and ultimately, neuronal cell death (Frank and Gupta, 2005). Aβ-induced-oxidative damages cause activation of the mitogenic activated protein kinase (MAP kinase) family and the nuclear factor kappa B (NF-κB) pathways which are the paramount signals in the neuronal cell death (Bai *et al.*, 2008; Chen *et al.*, 2008; Chongthammakun *et al.*, 2008). Several studies have demonstrated that inhibition of these pathways could be useful for the treatment of neurodegenerative diseases including AD (Munoz *et al.*, 2007; Paris *et al.*, 2007; Buggia-Prevot *et al.*, 2008; Wang *et al.*, 2008).

The brain is especially sensitive to oxidative stress due to its high concentration of readily oxidizeable fatty acids and high oxygen consumption. Antioxidants including green tea polyphenols are able to prevent or reduce the progression of AD (Weinreb *et al.*, 2004; Rezai-Zadeh *et al.*, 2008). Our studies have showed a protective effect of green tea extract on ischemia/reperfusion-induced brain injury and Aβ-induced PC12 cell death via an antioxidant mechanism (Hong *et al.*, 2000; Hong *et al.*, 2001; Lee *et al.*, 2004). We also reported the significance of MAPK and NF-κB activities in Aβ-induced neuronal cell death (Song *et al.*, 2004), and the inhibition of MAPK and NF-κB pathways by green tea components prevented Aβ-induced neuronal cell death in vitro and animal models (Lee *et al.*, 2005a; 2005b; Kim *et al.*, 2009).

Previous studies have demonstrated that L-theanine, an amino acid component of green tea, has anti-oxidative properties (Yokozawa and Dong, 1997; Cho *et al.*, 2008; Nishida *et al.*, 2008), and neuroprotective effects against ischemia and memory dysfunction (Kakuda, 2002; Egashira *et al.*, 2004; Nathan *et al.*, 2006; Egashira *et al.*, 2007; 2008; Yamada *et al.*, 2008). To further confirm whether the anti-oxidant property of L-theanine is significant for neuroprotective effects in neuronal cells, we investigated the inhibitory effect of L-theanine on

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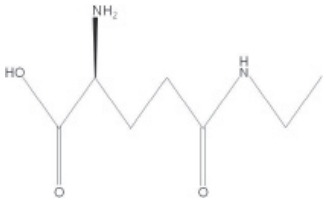

Fig. 1. The Chemical Structure of L-theanine (N-ethyl-L-Glutamine).

Ab-induced neurotoxicity and oxidative damages in cultured neuronal cell lines.

MATERIALS AND METHODS

L-theanine

L-theanine (Sunthanine), isolated from the leaves of Camellia Sinensis, was supplied as a white powder by Taiyo Kahaku Co., Ltd. (Yokkaichi, Japan). The purity was 100 ± 2%. The structure was shown in Fig. 1.

$A\beta_{1-42}$

The $A\beta_{1-42}$ (Sigma, St. Louis, MO; 2 μg/mouse) was dissolved and diluted in distilled water. The dissolved $A\beta_{1-42}$ was incubated for aggregation at 37°C for 4 days.

Cell culture

Human neuroblastoma cells (SK-N-SH, a dopaminergic cell; SK-N-MC, a nondopaminergic cell) were cultured in Dulbecco's modified Eagle's medium and F-12 nutrient supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C under an atmosphere of 5% CO_2 and 95% air.

Cell viability

The cells were plated in 96-well plates, and cell viability was determined by the conventional 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction assay (Sigma Chem. Co. St. Louis, MO). MTT assay measures the activity of intramitochondrial and extramitochondrial dehydrogenases. Briefly, tetrazolium salts are cleaved by dehydrogenases of viable cells to produce formazan, and the change of absorbance is detected spectrophotometrically. The cells were exposed with $A\beta_{1-42}$ at various concentrations (1-50 μM) with/without various concentrations of L-theanine (10-50 μg/ml) for 24 h to examine the recovery effect of L-theanine on $A\beta_{1-42}$-induced killing of cells. The cells were treated with the MTT solution (1 mg/ml) for 2 h. The absorbance was measured with a microplate reader (Tecan, sunrise, Salzburg, Austria) at 450 nm. Results were expressed as the percentage of MTT reduction.

Reactive oxygen species (ROS) generation

To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment with $A\beta_{1-42}$ (5 μM) for 72 h in the presence or absence of L-theanine (10-50 μg/ml), the cells were washed in modified Kreb's buffer containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 4 mM NaHCO3, 5.5 mM glucose and 10 mM Hepes (pH 7.4). The cell suspension was transferred into plastic tubes. Measurement was started by an injection of 5 μM DCF-DA in the dark. After 30 min of incubation at 37°C, generation was determined by Fluorometer (fmax, Molecular devices corp., San diego, CA) at Ex=485 nm and Em=538 nm.

Western blotting

Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chem. Co.), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 15,000×g for 15 min. Equal amount of proteins (40 μg) were separated on a SDS/10 and 15%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was then incubated for 3 h at room temperature with specific antibodies. Rabbit polyclonal antibodies against active form of JNK1 (1:500), ERK2 (1:500) and p38 MAPK (1:500), p65 (1:500) mouse polyclonal antibodies against phosphorylation forms of JNK1 (1:500), ERK2 (1:500) and p38 MAPK (1:500), and p50 (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used in this study. The blots were then incubated with the corresponding conjugated anti-rabbit, anti-mouse and anti-goat immunoglobulin G-horseradish peroxidases (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL western blotting detection system. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Com., Rochester, NY).

Nuclear extract and gel mobility shift assay

Gel mobility shift assay was done using a slight modification of a previously described method (Hong *et al.*, 2000). Briefly, the cultured cells were washed three times with ice-cold phosphate buffered saline (PBS, pH 7.6). The cell pellets were resuspended in 400 μl of cold buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged at 15,000×g for 6 min to remove everything except the nuclei. The pellets were resuspended in a second buffer containing 20 mM HEPES, 20% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF. After centrifugation at 15,000×g for 6 min, the supernatant contained the nuclear proteins. The protein level was determined by a microplate modification of the Bradford method (Bio-Rad Bulletin 1177, Bio-Rad Lab., Richmond, CA). The DNA binding activity of transcription factors was assayed according to the manufacturer's instructions (Promega Co., Madison, WI). In brief, 10 μg of nuclear protein was incubated in 25 μl total volume of incubation buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 4% glycerol, 0.08 mg/ml salmon sperm DNA) at 4°C for 15 min followed by another 20 min incubation with 250 μCi [γ-32P] ATP-labeled oligonucleotide containing NF-κB binding site at room temperature. The DNA binding activity of NF-κB was confirmed by the supershift assays using antibody of p65 and p50 as well as competition assay with unlabeled IκB oligonucleotide. The DNA-protein binding complex was run on a 6% non-denatured polyacrylamide gel at 150 volts for 2 h. Gels were dried and autoradio-

graphed using Kodak MR film at –80°C overnight.

Determination of carbonyl protein levels

Protein carbonyls in cultured neuronal cells were measured using the Cayman Protein Carbonyl assay kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. In brief, 0.1 g of cultured neuronal cells was homogenized with 1 ml of 50 mM MES containing 1 mM EDTA. After centrifugation at 10,000×g for 15 min at 4°C, 200 μl of supernatant of each sample was transferred to two 2 ml plastic tube of sample tube and control tube, added 800 μl of 10 mM 2,4-Dinitrophenylhydrazine (DNPH) to the sample tube and 800 μl of 2.5 M HCL to the control tube, and incubated in the dark at room temperature for 1 h with vortexing every 15 min. 1 ml of 20% TCA was added to each tube, vortexed and incubated on ice for 5 min. After centrifugation for 10 min at 10,000×g, the supernatant was discarded; the pellet was resuspended with 1 ml of 10% TCA and incubated on ice for 5 min. After centrifugation for 10 min at 10,000×g, the pellets were washed with 1 ml of ethanol-ethyl acetate (1:1) mixture, to remove any unreacted DNPH. After repeat the wash three times with 1 ml of ethanol-ethyl acetate (1:1) mixture, the pellets were solubilized with 500 μl of guanidine hydrochloride and centrifuged at 10,000×g for 10 min at 4°C to remove insoluble material. Carbonyl content was calculated from the absorbance measurement at 385 nm using microplate absorbance reader (Molecular Devices Corp., Sunnyvale, CA) and expressed as nmol/mg of protein.

Measurement of lipid peroxidation

As a measure of lipid peroxidation, the levels of hydroxynonenal-histidine (HNE-His) protein adducts were quantified by using the Oxiselect HNE-His Adduct ELISA Kit (Cell Biolabs, Inc., CA) according to the manufacturer's instructions. In brief, cells were homogenized with 1 ml of PBS containing 1% triton X-100. 100 μl of the 2 mg/ml protein sample was transferred to a 96-well protein binding plate, incubated for 4°C overnight and washed 2 times with 250 μl of 1X PBS per well. 200 μl of Assay per well was added to 96-well protein binding plate, incubated for 2 h at room temperature on an orbital shaker and washed three times with 250 μl of 1X Wash Buffer per well. 100 μl of the diluted anti-HNE-His antibody solution was added to all wells, incubated for 1 h at room temperature on orbital shaker, and washed again with 250 μl of 1X Wash Buffer per well. 100 μl of the secondary antibody-HRP conjugate solution was added to all wells, incubated for 1 h at room temperature on orbital shaker, and washed again with 250 μl of 1X Wash Buffer per well. After the final wash, 100 μl of substrate solution (TMB) was added to each well, was incubated for 20 min at room temperature. 100 μl of stopping reagent (0.5 M sulfuric acid) was added, and then HNE-his protein adduct was calculated from the absorbance measurement at 450 nm using microplate absorbance reader (Sunrise, Tecan, Switzerland) absorbance reader and expressed as pmol/mg of protein.

Measurement of total glutathione level

Total glutathione levels were determined using a glutathione assay kit (Cayman Chemical) according to the manufacturer's instructions. In brief, cells were homogenized with 1 ml of 50 mM MES containing 1 mM EDTA. After centrifugation for 15 min at 10,000×g, 1 ml of 10% metaphosphoric acid was added to the 1 ml of supernatant for deproteination, voltexed and incubated for 5 min at room temperature. After centrifugation for 2 min at 2,000×g, 50 μl of 4 M triethanolamine (Sigma Chem. Co.) per ml of supernatant was added to each sample. 50 μl of sample was transferred to the 96-well plate and 150 μl of assay cocktail (MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted 5; 5-Dithiobis (2-Nitrobenzoic Acid (0.45 ml)) were added to each well. After incubation for 30 min at room temperature, the GSH levels were calculated from the absorbance measurement at 405 nm microplate absorbance reader (Sunrise, Tecan, Switzerland) absorbance reader and expressed as nmol/mg of protein.

Statistical analysis

Statistical analysis of the data was carried out using analysis of variance (ANOVA) for repeated measures followed by Dunnette's post-hoc analysis using GraphPad Prism 4 soft-

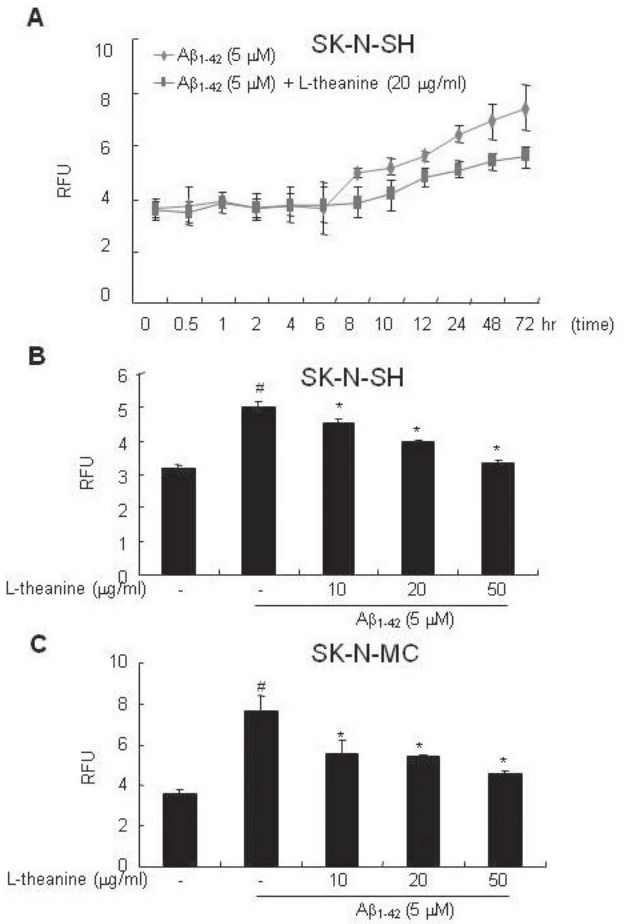

Fig. 2. Time dependent protective effect of L-theanine on $A\beta_{1-42}$-induced intracellular ROS in SK-N-MC cells (A), and dose dependent protective effect of L-theanine on $A\beta_{1-42}$-induced intracellular ROS in SK-N-MC and SK-N-SH cells (B, C). Intracellular ROS levels were determined by measuring DCF fluorescence. SK-N-SH and SK-N-MC cells were treated with the indicated concentrations of $A\beta_{1-42}$ (5 μM) or co-treated with L-theanine (10-50 μg/ml) for 72 h. Values are presented as mean ± S.D. from three independent experiments with duplicates. #Significant difference from untreated control (p<0.05). *Significant difference from $A\beta_{1-42}$-treated control (p<0.05).

ware (Version 4.03, GraphPad software, Inc.).

RESULTS

Effect of L-theanine on Aβ_{1-42}-induced reactive oxygen species generation in cultured neuroblastoma cells

Increased levels of intracellular reactive oxygen species (ROS) has been implicated in cell death. To determine whether L-theanine may attenuate cell death by blocking ROS generation, we measured intracellular ROS generation using a cell membrane permeable dye, DCF-DA. This dye is hydrolyzed to DCF which interacts with peroxides, forming fluorescent 2',7'-dichlorofluorescin. Treatment with Aβ_{1-42} (5 μM) for up to 72 hr increased intracellular ROS generation in a treatment time dependent manner in SK-N-MC cells, whereas L-theanine (20 μg/ml) reduced Aβ_{1-42}-induced intracellular ROS generation (Fig. 2A). Treatment with Aβ_{1-42} (5 μM) for 72 hr increased intracellular ROS generation whereas L-theanine (10-50 μg/ml) significantly reduced Aβ_{1-42}-induced intracellular ROS generation in a dose dependent manner in SK-N-SH (Fig. 2B) and SK-N-MC human neuroblastoma cells (Fig. 2C).

Effect of L-theanine on Aβ_{1-42}-induced decreased in glutathione levels, and increased carbonyl proteins, and lipid peroxidation products

Glutathione level were decreased from 15.5 ± 1.5 and 16.2 ± 1.6 in untreated SK-N-MC and SK-N-SH cells to 11.2 ± 1.2 and 11.1 ± 1.2 nmole/mg protein in Aβ_{1-42}-treated cells, respectively. This decrease in glutathione was recovered by L-theanine (50 μg/ml) treatment, returning glutathione levels to 14.3 ± 1.8 nmole/mg protein and 14.0 ± 1.8 nmole/mg protein in the both cells treated by L-theanine, respectively (Fig. 3, left panels). Inversely correlated with decreased glutathione levels, the level of lipid peroxidation products (4-hydroxynonenal) in the cells was increased from 0.58 ± 0.08 and 0.51 ± 0.09 to 0.81 ± 0.12, 0.79 ± 0.12, nmole/mg protein as a result of Aβ_{1-42} treatment. Neuronal cells pretreated with L-theanine (50 μg/ml) had substantially reduced lipid peroxidation product formation and 4-hydroxynonenal levels were 0.52 ± 0.05 and 0.43 ± 0.11 nmole/mg protein, respectively (Fig. 3, middle panel). The carbonyl protein level also increased in the cells treated with Aβ_{1-42} (1.70 ± 0.2 and 1.80 ± 0.2 nmole/mg protein) compared to the control cells (1.20 ± 0.01, 1.28 ± 0.08 nmole/mg protein). These values were reduced to 0.95 ± 0.30 and 0.81 ± 0.41 nmole/mg protein as a result of treatment with L-theanine (50 μg/ml) in both cells, respectively (Fig. 3, right panels).

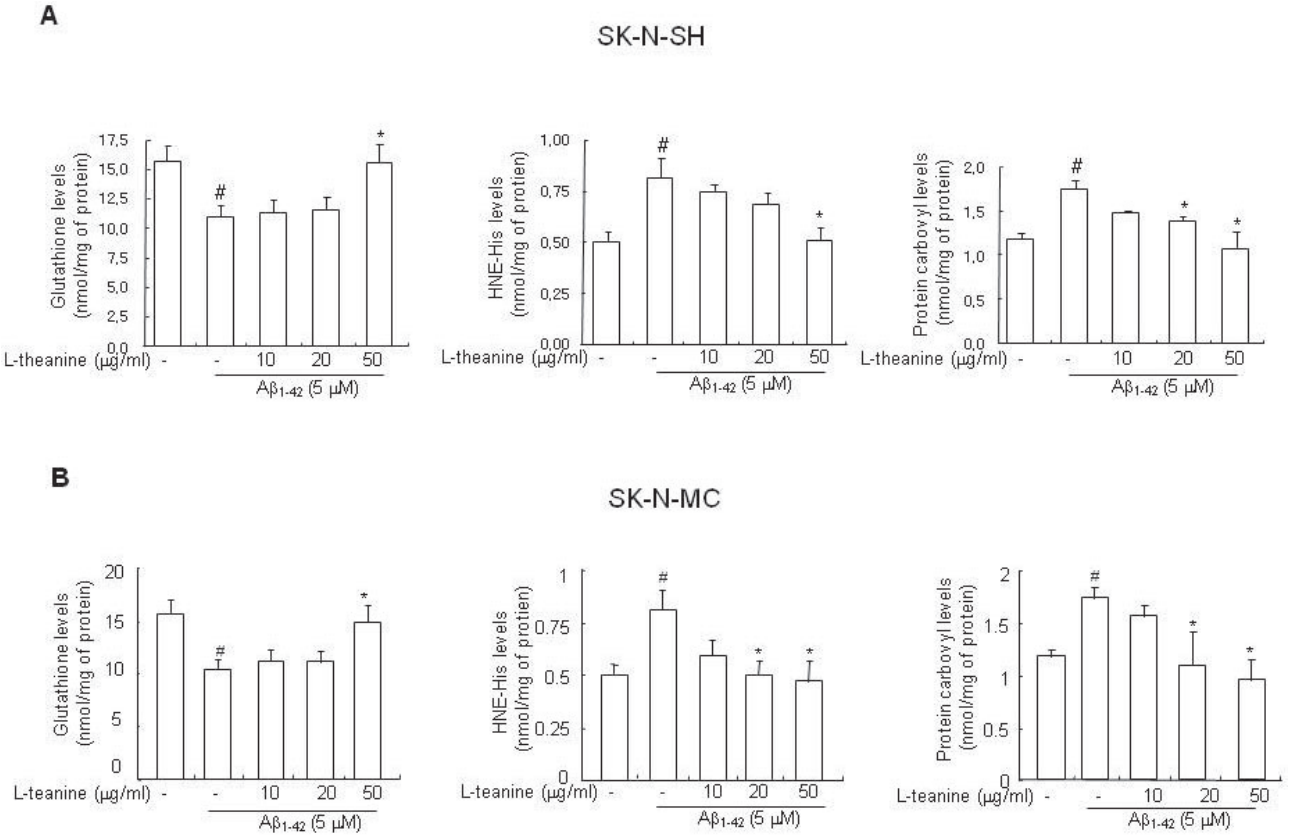

Fig. 3. Inhibitory effect of L-theanine on the Aβ_{1-42}-induced glutathione depletion, and generation of lipid peroxidation and carbonyl protein in SK-N-SH (A) and SK-N-MC (B) cells. SK-N-SH and SK-N-MC cells were treated with the indicated concentrations of Aβ_{1-42} (5 μM) or co-treated with L-theanine (10-50 μg/ml) for 72 h. Values are presented as mean ± S.D. from three independent experiments with duplicates. #Significant difference from untreated control (p<0.05). *Significant difference from Aβ_{1-42}-treated control (p<0.05).

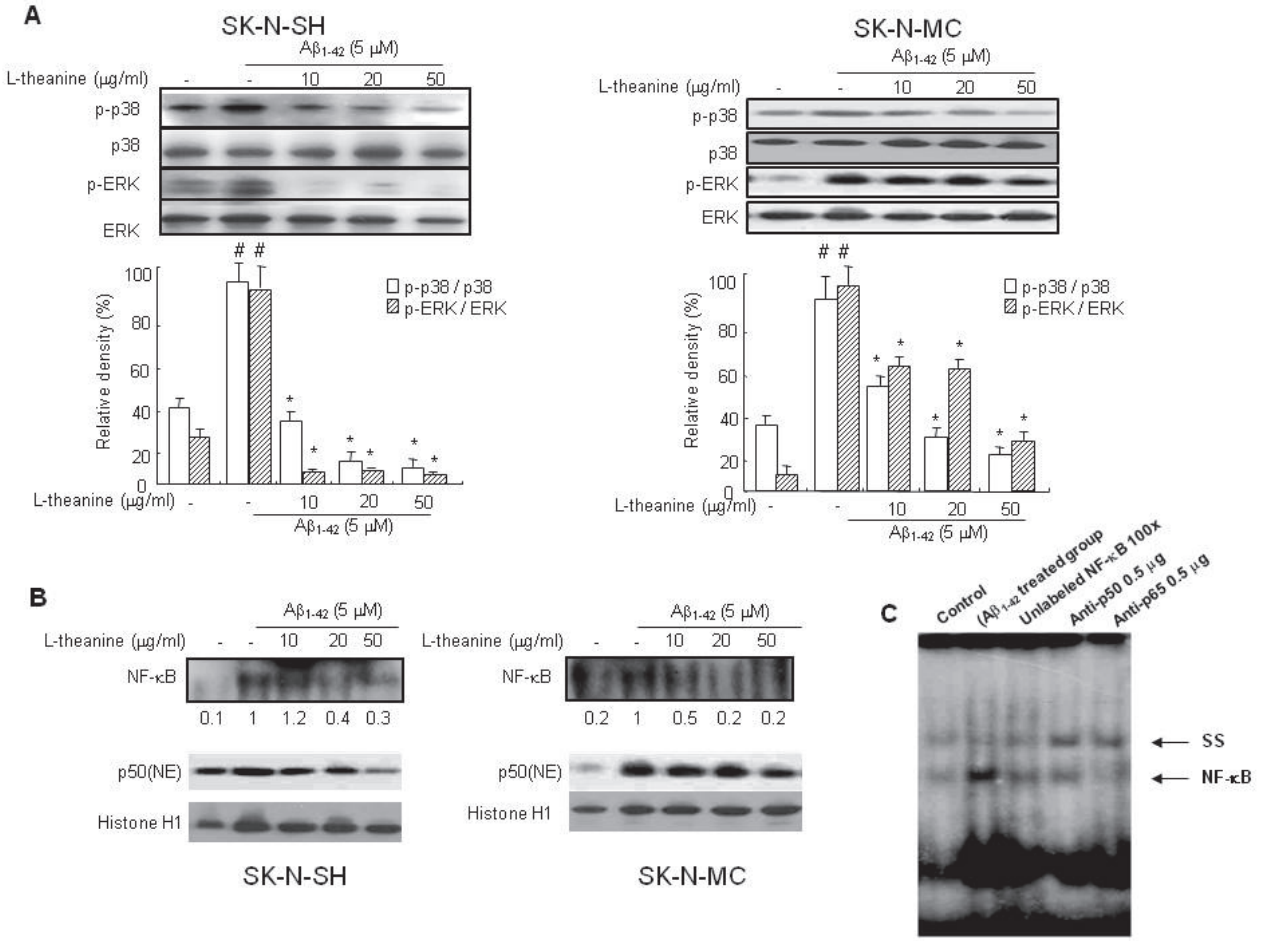

Fig. 4. The inhibitory effect of L-theanine on $A\beta_{1-42}$-induced activation of MAP kinase (A) and NF-κB (B) in the cultured SK-N-SH and SK-N-MC cells. The effect of L-theanine on the cytoplasmic levels of phosphorylated MAP kinases. SK-N-SH and SK-N-MC cells were treated with the indicated concentrations of $A\beta_{1-42}$ (5 μM) or co-treated with L-theanine (10-50 μg/ml) for 72 h. Immunoblots of cytoplasmic lysates from cultured cells were then probed with antibodies against MAP kinases, and were determined by western blotting as described in materials and methods (A). NF-κB activation was determined by EMSA in the nuclear extract of cells as described in the materials and methods (B). Densitometric values are the means ± S.D. of three independent experiments with duplicates (A, B). #Significant difference from untreated control (p<0.05). *Significant difference from $A\beta_{1-42}$ treated control (p<0.05). The DNA binding activity of NF-κB was confirmed by the competition and supershift assays in SK-N-SH (C).

Effect of L-theanine on the $A\beta_{1-42}$-induced activation of ERK/p38, MAPK, and NF-κB

Since the activation of MAPK is implicated in oxidative stress-induced cell death and the regulation of NF-κB activation, we investigated the effect of L-theanine on the MAPK activation. As shown in Fig. 4A, $A\beta_{1-42}$ (2 μg/mouse) induced ERK and p38 activation (increased expressions of phosphorylated ERK1/2 and p38), while L-theanine blocked it in a dose dependent manner (Fig. 4A), but JNK pathway was not changed. NF-κB activation was investigated since NF-κB is also known to be implicated in oxidative stress-induced neuronal cell death. $A\beta_{1-42}$ caused an increase in NF-κB DNA binding activity in the both cells which was inhibited by L-theanine in a dose dependent manner in the both neuronal cells (Fig. 4B). $A\beta_{1-42}$ induced translocation of p50 into the nucleus was significantly decreased (Fig. 4B). The DNA binding activity of NF-κB was confirmed by the supershift and competition assays (Fig. 4C).

Effect of L-theanine on $A\beta_{1-42}$-induced cell growth of cultured neuroblastoma cells

Increased levels of intracellular reactive oxygen species (ROS) has been implicated in cell death. To determine whether L-theanine may attenuate cell growth inhibition by blocking ROS generation, cell viability was determined. $A\beta_{1-42}$ treatment time dependent decreased cell viability for up to 72 hr, but reversely consistent with the ROS generation, L-theanine treatment (20 μg/ml) recovered $A\beta_{1-42}$-decreased cell viability in SK-N-MC cells (Fig. 5A). In further dose dependent study, L-theanine treatment (10-50 μg/ml) substantially recovered $A\beta_{1-42}$-decreased cell viability in a dose dependent manner in both SK-N-SH and SK-N-MC cells (Fig. 5B).

DISCUSSION

Our present study showed that L-theanine, a major peptide

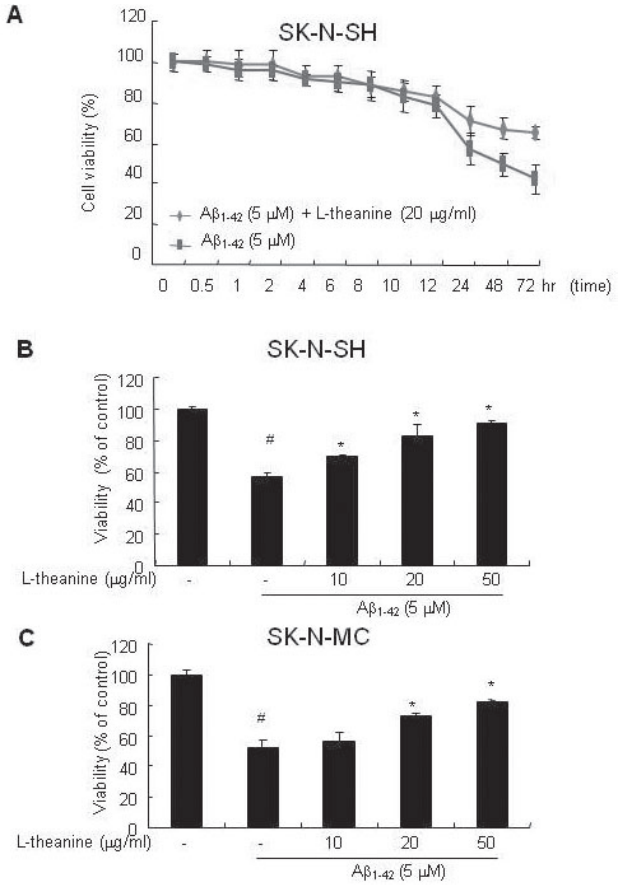

Fig. 5. Time dependent protective effect of L-theanine on $A\beta_{1-42}$-induced cytotoxicity in SK-N-MC cells (A), and dose dependent protective effect of L-theanine on $A\beta_{1-42}$-induced cytotoxicity in SK-N-MC and SK-N-SH cells (B, C). Protective effect of L-theanine on $A\beta_{1-42}$-induced cytotoxicity was determined by MTT assay. SK-N-SH and SK-N-MC cells were treated with the indicated concentrations of $A\beta_{1-42}$ (5 μM) or co-treated with L-theanine (10-50 μg/ml) for 72 h. Values are presented as mean ± S.D. from three independent experiments with duplicates. #Significant difference from untreated control (p<0.05). *Significant difference from $A\beta_{1-42}$-treated control (p<0.05).

component of green tea, reduces $A\beta_{1-42}$-induced neurotoxicity through a protective effect against oxidative damages. Our data also showed that inhibition of p38/ERK1/2 and NF-κB pathway signals may contribute to the inhibition of neuronal cell damages. Several our previous studies and those of others have shown that green tea and its components possess neuroprotective effects on several neurodegenerative diseases including middle cerebral artery occlusion-induced ischemia (Kakuda, 2002; Egashira *et al.*, 2004; Egashira *et al.*, 2007) as well as Alzheimer's diseases (Nathan *et al.*, 2006; Egashira *et al.*, 2008; Yamada *et al.*, 2008; Kim *et al.*, 2009) through anti-oxidant mechanisms (Hong *et al.*, 2000; Hong *et al.*, 2001; Mandel *et al.*, 2004; Lee *et al.*, 2004). It is generally well known that ROS by Aβ is critical factors for the formation of senile plaques in the brains of AD patients (Milton, 2004; Chen and Yan, 2007; Shibata and Kobayashi, 2008). In present study, theanine prevented Aβ-induced ROS generation and oxidative damages of macromolecules such as protein (carbonyl protein level) and lipid (HNE-His level), but recovered the Aβ-induced reduction of the glutathione level. In agreement with these our data, we also found that L-theanine prevented oxidative damages macromolecules in the brain of an AD mice model (Kim *et al.*, 2009). Thus, inhibitory effects of L-theanine on $A\beta_{1-42}$-induced ROS generation and oxidative damages could be significant in the inhibitory effect of L-theanine on $A\beta_{1-42}$-induced neurotoxicity. In fact, we observed that conversely related to the lowering effect of L-theanine on the $A\beta_{1-42}$-induced elevation of ROS and oxidative stress, L-theanine prevented $A\beta_{1-42}$-induced neurotoxicity in a dose dependent manner. These data indicate that L-theanine could be also an important peptide component of green tea for prevention of development or progression of AD.

L-theanine inhibited $A\beta_{1-42}$-induced activation of NK-κB, a redox-regulated transcription factor. NF-κB can be activated via induction of intracellular ROS (Hong *et al.*, 2000; Valerio *et al.*, 2006). Therefore, the inhibitory ability of L-theanine on ROS generation could result in inactivation of NF-κB induced by $A\beta_{1-42}$. Agreed with present findings, we previously found that $A\beta_{25-35}$-increased NF-κB was prevented by green tea extract in cultured PC-12 cells (Lee *et al.*, 2005) as well as in the $A\beta_{1-42}$-infused brain of AD model mice (Kim *et al.*, 2009; Lee et al., 2009). NF-κB is implicated in the neuronal cell death in the development of Alzheimer's disease and other neurodegenerative disease, and several antioxidants have been known to prevent the progreesion and development of these disease through inhibition of NF-κB (Mattson and Meffert, 2006; Plesnila *et al.*, 2007; Lee *et al.*, 2009; Tusi *et al.*., 2010). Therefore, it is possible that the ability of L-theanine to prevent ROS generation could be related to its inhibitory effect on Aβ-induced neurotoxicity through inhibition of NF-κB. It is also hypothesized that L-theanine may block the activation or/and expression of ROS-activated upstream signaling pathways. This hypothesis was supported by the finding that L-theanine prevented the $A\beta_{1-42}$-induced activations of ERK1/2 and/or p38 MAPK, which are upstream signaling pathways of NF-κB. Very similar to our finding, it was also found that L-theanine attenuated both rotenone- and dieldrin-induced DNA fragmentation and apoptotic death in SH-SY5Y cells through prevention of rotenone- and dieldrin-induced heme oxygenase-1 (HO-1) up-regulation and (ERK1/2) in SH-SY5Y cells (Cho *et al.*, 2008). Meanwhile, the activation of c-Jun N-terminal kinase and caspase-3 induced by l-glutamate was suppressed by l-theanine in swedish mutation SH-SY5Y cells which over-generated Aβ. These results indicate that the ROS scavenging ability of L-theanine may be associated with the inhibition of the ERK1/2 and/or p38 MAPK pathways. Other mechanisms could be involved. Direct removing of Aβ by activation of could be a possible since theanine has endopeptidase activity (Ayoub and Melzig, 2006). However, theanine may not react directly with Aβ since we did not observe disruption of the aggregation of Aβ (data not shown). Taken together, these data suggest that L-theanine could be useful in the treatment of AD. This is due to its ability to inhibit neurotoxicity via an inhibition of both Aβ-induced oxidative stress, and activation of ERK1/2/p38 MAPK and NF-κB pathways.

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