Protective effect of the green tea component, L-theanine on environmental toxins-induced neuronal cell death

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1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. It is characterized by a selective and progressive degeneration of substantia nigra (SN) dopaminergic (DA) neurons (Nagatsu and Sawada, 2007). Although, the exact mechanisms of nigral DA neuronal degeneration remains unknown, postmortem studies showed that dying cells bear the signs of apoptosis, in particular chromatin condensation, DNA fragmentation, oxidative damage, mitochondrial dysfunction, and caspase activation (Nagatsu and Sawada, 2007; Olanow and Tatton, 1999).

Increasing evidences from epidemiological studies have implicated that a variety of environmental factors, especially pesticide exposure and sequential oxidative stress, play an important role in some sporadic cases of PD (Chun et al., 2001; Singh et al., 2007). Among the identified PD risk factors, rotenone is a naturally occurring plant compound and a common insecticide has long been used. It has been known to produce neuropathological changes and clinical features similar to idiopathic PD (Shamoto-Nagai et al., 2003; Sherer et al., 2007). Another organochlorine pesticide, dieldrin also has been proposed as a possible neurotoxin to cause PD or PD-like symptoms (Kanthasamy et al., 2005). Recent studies have shown that these PD-related neurotoxins are accumulated in brain tissues and cause toxic effects to monoaminergic and DA neurons (Betarbet et al., 2000; Corrigan et al., 2000). Of interest those PD-related neurotoxins caused oxidative stress via elevation of reactive oxygen species (ROS) (Kanthasamy et al., 2005; Shamoto-Nagai et al., 2003). Moreover, several studies demonstrated that increased oxidative stress and defective mitochondrial oxidative phosphorylation are more common in PD patients than in normal controls (Keeney et al., 2006; Schapira, 2008). Consistent with these findings, much interest has focused on the antioxidants that may be promising therapeutics for PD.

It has been known that green tea (Camellia sinensis) leaves contain potential antioxidant compounds such as L-theanine, caffeine, and various catechins (Chen et al., 2003). Among those...
chemical components in green tea, L-theanine is a natural amino acid structurally similar to glutamate and is a characteristic flavourous component of tea. Recent studies evidenced that L-theanine has neuroprotective effects on the ischemic brain damage and glutamate-induced cell death in cortical neurons (Egashira et al., 2004; Kakuda et al., 2002). Furthermore, other studies reported that L-theanine could easily cross the blood–brain barrier, and acts in the brain (Yokogoshi et al., 1998a,b). On the other hand, the effect of L-theanine against the pathogenesis of PD has not been studied. Therefore, we examined the neuroprotective effects of L-theanine against rotenone- or dieldrin-induced DA neuronal damage. In this study, we found for the first time that L-theanine-mediated neuroprotection in DA neurons was involved in the attenuation of apoptotic cell death and modulation of HO-1, ERK1/2, and neurotrophic factors.

2. Materials and methods

2.1. Cell culture and treatments

The human DA neuronal cell line, SH-SY5Y, was cultured in DMEM/F12 medium (Life Technologies, Rockville, MD, USA) supplemented with 10% FBS and penicillin (100 units/ml)-streptomycin (100 μg/ml) at 37 °C in 5% CO2. Usually, 1 day before any treatment, the culture medium was changed to DMEM/F12 medium with 0.5% FBS to reduce the serum effect. When indicated, L-theanine was added 1 h prior to the treatment of rotenone or dieldrin. In a single experiment each treatment was performed in triplicate.

2.2. Cell death assay

To estimate cell viability, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay was used in combination with the total cell counting, using trypan blue dye exclusion as previously described (Chun et al., 2001). Nuclear staining with DAPI (4,6-diamidino-2-phenylindole) was performed to evaluate apoptosis. SH-SY5Y cells were cultured in 24-well plates at a seeding density of 1 × 104 cells per well for 24 h, and then treated with rotenone or dieldrin for indicated periods pretreated with or without L-theanine for 1 h. The treated SH-SY5Y cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. Permeate the cells with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed cells were stained with DAPI (300 nM) for 5 min at room temperature in dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71, Olympus, Japan). Percentage of apoptotic cells, which coincided with morphological criteria of apoptosis such as DNA fragmentation, nuclear condensation, and segmentation, was counted in the total number of cells.

2.3. Immunoblot analysis

SH-SY5Y cells grown under various experimental conditions were washed twice with phosphate-buffered saline (PBS), and then lysed with RIPA buffer (PBS, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 30 mg/ml aprotinin and 1 mM Na3VO4). Proteins were separated on 10–15% SDS–PAGE and blotted onto PVDF membrane. The membrane was treated with primary antibody for Caspase-3 (1:1000 dilution; Cell Signaling Technology, USA), HO-1 (1:4000 dilution; Stressgen, USA), p-ERK1/2 (1:1000 dilution; Cell Signaling Technology, USA), ERK1/2 (1:1000 dilution; Cell Signaling Technology, USA), or actin (1:4000 dilution; Santa Cruz Biotechnologies, USA), and then reacted with HRP-conjugated secondary antibody. Immunoreactive bands were detected by ECL chemiluminescence kit (GE Healthcare, USA).

2.4. Measurement of neurotrophic factors by ELISA

To evaluate the effect of neurotrophic factors release, the SH-SY5Y cells were stabilized for 24 h in DMEM/F12 medium and the media were replaced with serum deprived medium in the presence of L-theanine and each PD-related neurotoxicansts at 37 °C in 5% CO2. Conditioned media were collected after 24 h and stored at −80 °C until assayed by ELISA. The amount of neurotrophic factors (BDNF, GDNF and NT-4) in each conditioned medium was quantified using Promega's Emax ImmunoAssay Systems and the automatic ELISA plate reader.

2.5. Statistical analysis

The data were expressed as the mean ± S.E.M. Data were first analyzed using one-way factorial analysis of variance (ANOVA). Student’s t-test or Turkey’s test was then performed to compare treated samples, and p < 0.05 was considered significant.

3. Results

3.1. Protective effect of L-theanine against PD-related neurotoxicanst

In this study, the effect of L-theanine on rotenone- or dieldrin-induced SH-SY5Y cell viability loss was assessed by XTT assay and with trypan blue exclusion test. Initial studies were performed to examine the cytotoxic response of SH-SY5Y cells to L-theanine. Cells were treated with various concentrations of L-theanine (125–2000 μM) for 24 h. As shown in Fig. 1a, when exposed to 500 μM or lower concentration of L-theanine, viability of SH-SY5Y cells was same as untreated control cells. Furthermore, 1 mM or higher concentration of L-theanine treatment did not significantly affect the cell viability of SH-SY5Y cells. Thus, we did subsequent experiments using 500 μM of L-theanine.

Previous studies showed that SH-SY5Y cells were treated with rotenone at concentrations ranging from 50 nM to 50 μM according to the initial seeding density and treatment periods (Chung et al., 2007; Hsuan et al., 2006). In our experimental conditions, the effect of rotenone on SH-SY5Y cell death was detectable at concentrations of 1–10 μM after 24 h treatment (Fig. 1b). In addition, dieldrin caused cytotoxicity in SH-SY5Y cells at concentrations ranging from 5 to 40 μM. Based on the LD50 dose–response data from Fig. 1b, 5 μM rotenone and 10 μM dieldrin were chosen for our subsequent experiments.

To evaluate the effect of L-theanine on PD-related neurotoxicanst-induced DA cell death, SH-SY5Y cells were pretreated with 500 μM L-theanine for 1 h, followed by 5 μM rotenone or 10 μM dieldrin treatment for 24 h. As shown in Fig. 1c, PD-related neurotoxicanst-induced loss of cell viability was significantly attenuated by L-theanine treatment.

3.2. L-Theanine prevented PD-related neurotoxicanst-induced changes in nuclear morphology and Caspase-3 activation

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 2, the control SH-SY5Y cell’s nuclei had a normal regular and oval shape. The nuclear morphology of cells exposed to L-theanine alone was intact and similar to that of untreated control cells. However, treatment with 5 μM rotenone or 10 μM dieldrin for 24 h resulted in nuclear condensation and fragmentation, characteristics of apoptosis. In contrast, L-theanine pretreatment significantly blocked the rotenone- or dieldrin-induced nuclear damage.

Activation of Caspase-3 has been implicated in dopaminergic apoptotic cell death (Hartmann et al., 2000). Therefore, we
determined whether these events occurred during human dopaminergic cell death in our model by measuring the inactive full length Caspase-3 (35 kDa) and the active cleaved fragment (17 kDa) by Western blot analysis.

Incubation of SH-SY5Y cells with rotenone (5 μM) or dieldrin (10 μM) induced the cleavage of Caspase-3 from its native 35 kDa form to an active 17 kDa fragment, indicative of cells undergoing apoptosis. However, the cells pre-incubated for 1 h with L-theanine prior to the addition of rotenone or dieldrin, showed a marked attenuation of Caspase-3 activation (Fig. 3). These suggest that the protective effect of L-theanine is mediated via an, at least in part, anti-apoptotic pathway.

3.3. L-Theanine attenuated PD-related neurotoxins-induced HO-1 elevation

Several studies suggested that dieldrin and rotenone induced oxidative damage in dopaminergic system (Kanthasamy et al., 2005; Shamoto-Nagai et al., 2003). Because HO-1 induction is the most sensitive cellular marker for oxidative stress (Schipper et al., 1998), we examined whether L-theanine has any effect on the expression of HO-1 in dieldrin- or rotenone-treated cells using Western blot analysis. As shown in Fig. 4, treatment of SH-SY5Y cells with rotenone (5 μM) or dieldrin (10 μM) led to significant increase in HO-1 expression. However, L-theanine (500 μM) pretreatment showed a marked attenuated expression of HO-1 as compared with the PD-related neurotoxins treatment alone. This suggests that L-theanine could suppress, at least in part, the oxidative stress in dopaminergic neurons.

3.4. L-Theanine blocked PD-related neurotoxins-induced reduction of ERK1/2 activation

Although the exact mechanisms by which flavonoids exert their neuroprotective actions remain unclear, it has been shown recently that mitogen-activated protein kinase (MAPK) signaling cascades are related to the effect of flavonoids (Schroeter et al., 2003).
The MAPK family consists of both apoptotic and anti-apoptotic proteins and the balance between these proteins is critical to neuronal survival and death. It has been known that ERK1/2 MAPK is an anti-apoptotic molecule whereas JNK and p38 MAPKs are pro-apoptotic proteins (Schroeter et al., 2002). In this study, we investigated whether L-theanine has any effect on the expression of ERK1/2 in dieldrin- or rotenone-treated cells using Western blot analysis. As shown in Fig. 5, ERK1/2 activation decreased significantly in 5 μM rotenone or 10 μM dieldrin-treated cells compared with that in control. However, L-theanine (500 μM) pretreatment could keep the ERK1/2 activation level almost to the normal values. Because L-theanine pretreatment did not significantly change the level of JNK and p38 activation (data not shown), our results suggest that ERK1/2 activation is likely critical for neuroprotection by L-theanine.

### 3.5. Neurotrophic factors release by L-theanine

To investigate the neurotrophic factor levels after the treatment of L-theanine and PD-related neurotoxins in human dopaminergic...
neurons, GDNF, BDNF and NT-4 levels were examined using Emax ImmunoAssay system (Promega, USA). Fig. 6 shows the response to PD-related neurotoxins (rotenone and dieldrin) and neuroprotectant (\(\delta\)-theanine) in the SH-SYSY cells. All those neurotoxins inhibited GDNF and BDNF release by 50–80%. However, NT-4 release in SH-SYSY cells was not affected by any of those neurotoxics or neuroprotectant (data not shown). Interestingly, \(\delta\)-theanine pretreatment significantly attenuated the down-regulation of GDNF and BDNF release.

4. Discussion

Some recent studies suggested that environmental factors appear to play a significant role in the typical, non-familial PD (Corrigan et al., 2000; Singh et al., 2007). Exposure to pesticides such as rotenone and dieldrin has been consistently reported to be associated with increased risk of PD. Although the causal mechanisms by which rotenone and dieldrin cause DA neuronal degeneration are not fully understood, a common denominator in the pathogenesis by rotenone and dieldrin is the involvement of oxidative stress-mediated apoptotic process (Kanathasamy et al., 2005; Shamoto-Nagai et al., 2003; Sherer et al., 2007). Therefore, it is important to examine cell death mechanisms by those pesticides and identify potential neuroprotectants for PD treatment.

Recently, naturally occurring components such as phytochemicals have received great attention because they are perceived as safe and functional compounds to treat the neurodegenerative disorders. We paid special attention to the \(\delta\)-theanine contained in green tea due to its neuroprotective effects (Egashira et al., 2004; Kakuda, 2002; Kakuda et al., 2002). \(\delta\)-Theanine is a naturally occurring amino acid component found in high-grade Japanese green tea (C. sinensis). It has been known that \(\delta\)-theanine has multiple biological activities, such as anti-stress effects (Kimura et al., 2007), suppressive effect against the stimulatory action of caffeine (Kakuda et al., 2000), and the effect of reducing systemic blood pressure (Yokogoshi et al., 1995). \(\delta\)-Theanine could pass through the blood-brain barrier and transported into the brain fairly rapidly after administered orally in animal experiments (Yokogoshi et al., 1998a,b). Recently, it has been proposed that \(\delta\)-theanine has neuroprotective effects against ischemia-induced neuronal death in hippocampal CA1 region and glutamate-induced cell death in cultured rat cortical neurons (Kakuda; 2002; Kakuda et al., 2000, 2002). Furthermore, administration of \(\delta\)-theanine enhanced the concentration of dopamine in the adult brain (Yokogoshi et al., 1998a) and enhanced synthesis of neurotrophic factors and neurotransmitters during a nerve-maturing period in the infant brain (Yamada et al., 2007). However, as far as we are aware, there are no studies on the effects of \(\delta\)-theanine on PD. In this study, we found that \(\delta\)-theanine could modulate PD-related neurotoxins-induced cell death in human DA neurons. A number of possible evidences indicated apoptotic cell death in the SN of PD patient (Anglade et al., 1997; Nagatsu and Sawada, 2007). In accordance with those previous studies, our results showed the essential morphologic form of apoptosis and Caspase-3 activation when treated with rotenone or dieldrin in SH-SYSY cells (Figs. 2 and 3). Of interest \(\delta\)-theanine pretreatment significantly attenuated the chromatin condensation, nuclear fragmentation and Caspase-3 activation. From these results, it is clear that \(\delta\)-theanine provides some protection against the apoptotic cell death by PD-related neurotoxins. However, it is at present unclear whether there is a direct interaction between \(\delta\)-theanine and PD-related neurotoxins in the DA cell lysates.

Heme oxygenase-1 (HO-1) is a cellular stress protein expressed in brain and other tissues in response to oxidative challenge (Schipper et al., 1998). HO-1 is a 32-kDa member of the stress response protein superfamily that catalyses the rapid degradation of heme to biliverdin in brain and other tissues (Ewing and Mainess, 1991; Schipper et al., 1998). Biliverdin, the bile pigment, is subsequently converted to bilirubin, which is a potent endogenous anti-oxidant. It was demonstrated that the expression of the HO-1 was up-regulated in various cells by oxidative stress, focal ischemia, and glutamate excitotoxicity (Kim et al., 2005; Nimura et al., 1996; Parfenova et al., 2006). On the other hand, heme's degradation process can increase free iron and carbon monoxide that can damage the mitochondrial membrane and induce oxidative stress under certain circumstances (Ewing and Mainess, 1991; Zhang and Plantadosi, 1992). This means that HO-1 up-regulation can induce a cytoprotective effect in neurodegenerative diseases, or, on the contrary, it accelerates neuronal cell damage. In this study, we found that \(\delta\)-theanine could partially attenuate the HO-1 expression. This result proposes that \(\delta\)-theanine can suppress the ROS production and subsequently attenuate the HO-1 expression.

Some dietary agents including tea components have been suggested as potent modulators of intracellular signal transduction (Schoereter et al., 2002). Therefore, we examined if any major intracellular signaling MAPK cascade is modulated by \(\delta\)-theanine. Recent studies have demonstrated that rotenone and dieldrin enhanced the production of ROS in neurons (Kanathasamy et al., 2005; Shamoto-Nagai et al., 2003). Furthermore, accumulating evidences indicated that the ROS increase not only inflicts direct cellular damage, but also acts as a major modulator for MAPK signaling cascades (Schoereter et al., 2002). There are at least four MAPK subfamilies, ERK1/2, JNK, p38 MAPK, and ERK5. It has been known that ERK1/2 may promote neuronal survival and contribute to the maintenance of mature DA neurons (Hsuann et al., 2006; Schoereter et al., 2002). However, JNK and p38 MAPK signaling pathways have been suggested to be a key trigger of apoptosis. Previous studies suggested that JNK and p38 are required for rotenone-induced apoptosis in SH-SYSY cells, but activation of ERK1/2 effectively blocked rotenone-induced cell death (Hsuann et al., 2006; Newhouse et al., 2004). In addition, JNK but not ERK1/2 or p38 plays a role in dieldrin-induced loss of cultured DA neurons (Chun et al., 2001). Of interest rotenone and dieldrin preferentially attenuated the activation of ERK1/2 in SH-SYSY cells (Fig. 4). However, pretreatment of SH-SYSY cells with \(\delta\)-theanine retained phosphorylation of ERK1/2. Although rotenone or dieldrin treatment induced phosphorylation of JNK and p38, \(\delta\)-theanine did not significantly change the activated level of those MAP kinases (data not shown). These results indicate that \(\delta\)-theanine protects DA cells through the ERK1/2 signaling pathway, whereas JNK and p38 activity is not affected in response to \(\delta\)-theanine. Gliarial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) act as a potent and specific neurotrophic factors for midbrain dopaminergic neurons (Siegel and Chauhan, 2000). It has been known that GDNF and BDNF exert both protective and reparative effects on the nigrostriatal dopamine system in animal models of PD (Jenner and Olanow, 1998). Importantly, the levels of neurotrophic factors such as BDNF, GDNF, basic fibroblast growth factor (bFGF), and ciliary neurotrophic factor (CNTF) are decreased in the SN of PD patients (Howells et al., 2000; Jenner and Olanow, 1998; Siegel and Chauhan, 2000; Tooyama et al., 1993). It has been reported that various molecules could regulate the expression of BDNF and GDNF in DA neurons. The transcription factor Pitx3 increased the mRNA levels of BDNF and GDNF in SH-SYSY cells and primary ventral mesencephalic cultures (Peng et al., 2007). Our previous studies demonstrated that some molecules, such as glutamate, TNF-\(\alpha\), cholecystokinin, BMP-4, cGMP and salicylic acid promoted BDNF production, and others including potential free radical.
Theanine protects human DA cells against rotenone- or dieldrin-induced cytotoxicity. Theanine significantly attenuated morphological changes of nuclei and HO-1 elevation which is related to overproduction of ROS, and partially blocked the decrease in ERK1/2 activation and the release of BDNF and GDNF in rotenone- or dieldrin-treated SH-SY5Y cells. This study may open up a new clinical strategy in progressive neurodegenerative diseases such as Parkinson’s disease though further research into L-theanine’s neuroprotective mechanisms will be necessary.

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References


