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L-Theanine attenuates cadmium-induced neurotoxicity through the inhibition of oxidative damage and tau hyperphosphorylation

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ABSTRACT

Cadmium (Cd) has long been known to induce neurological degenerative disorders. We studied effects of L-theanine, one of the major amino acid components in green tea, on Cd-induced brain injury in mice. Male ICR mice were intraperitoneally injected with L-theanine (100 or 200 mg/kg/day) or saline and after one hour these mice were orally administrated with $CdCl_2$ (3.75–6 mg/kg). The treatment was conducted for 8 weeks. L-Theanine significantly reduced Cd level in the mouse brain and plasma. Cd-induced neuronal cell death in the mouse cortex and hippocampus were apparently inhibited by L-theanine treatment. L-Theanine also decreased the levels of malondialdehyde (MDA) and ROS, and obviously elevated the levels of glutathione (GSH) and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in the mouse brain. Hyperphosphorylation of tau protein is proposed to be an early event for the evolution of tau pathology, and may play an important role in Cd-induced neurodegeneration. Our results showed that L-theanine significantly suppressed Cd-induced tau protein hyperphosphorylation at Ser199, Ser202, and Ser396. Mechanism study showed that L-theanine inhibited the activation of glycogen synthase kinase-3β (GSK-3β) which contributed to the hyperphosphorylation of tau and Cd-induced cytotoxicity. Furthermore, L-theanine reduced Cd-induced cytotoxicity possibly by interfering with the Akt/mTOR signaling pathway. In conclusion, our study indicated that L-theanine protected mice against Cd-induced neurotoxicity through reducing brain Cd level and relieved oxidative damage and tau hyperphosphorylation. Our foundings provide a novel insight into the potential use of L-theanine as prophylactic and therapeutic agents for Cd-induced neurodegenerative diseases.

1. Introduction

The heavy metal Cadmium (Cd) is widely distributed in natural and industrial sources. Populations are exposed to Cd through intake of contaminated food or water, or by inhalation of polluted air or tobacco smoke (Wang and Du, 2013). Basically there are three possible ways for human to absorb Cd, which includes gastrointestinal, pulmonary and dermal (Godt et al., 2006). Because of its long biological half-life (10–30 years in humans), Cd may injure major organs including liver, kidney, lung, testis and brain (Siu et al., 2009; Torra et al., 1995). Cd increases blood-brain barrier permeability, and induces brain injury via triggering oxidative damage to critical biomolecules, such as thiols, lipids, proteins and DNA (Figueiredo-Pereira et al., 1998; Lopez et al., 2006; Méndez-Armenta et al., 2003; Torra et al., 1995). Cd toxicity is associated with neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Barnham and Bush, 2008; Panayi et al., 2002). The pathological hallmark of AD is the neurofibrillary tangles (NFTs) and aggregates of paired helical filaments (PHFs). PHFs are assembled from hyperphosphorylated forms of the

Abbreviations: Cd, cadmium; AD, Alzheimer disease; PD, Parkinson disease; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; LPO, lipid peroxidation; MDA, malondialdehyde; $O_2^{\bullet}-$, superoxide anion; $^{\bullet}OH$, hydroxyl radicals; H_2O_2, hydrogen peroxide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DTNB, 5,5-dithio-bis-2-nitrobenzoic acid; DAPI, 4′ 6′-diamidino-2-phenylindole dihydrochloride; MT, microtubule; GSK-3β, glycogen synthase kinase; PI3K, phosphoinositide 3′-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; p70S6K1, ribosomal p70 S6 kinase.

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microtubule-associated protein tau (Alonso et al., 1996). Hyperphosphorylated tau protein promotes the instability of microtubule (MT) and exerts a neurotoxic effect such as chromatin condensation, DNA fragmentation, and caspase-3 activation, and finally results in neurodegeneration (Fath et al., 2002). Therefore, inhibition of tau protein hyperphosphorylation has become a hot spot for therapeutic intervention in tau pathology. Wang et al. found that Cd induced tau hyperphosphorylation and resulted in the destruction of MTs (Wang et al., 2016). Glycogen synthase kinase-3β (GSK-3β) is a major tau kinase involved in tau protein hyperphosphorylation (Rankin et al., 2007). Chronic Cd exposure could lead to intracellular ROS accumulation, which disrupts cellular redox regulatory systems and activates signaling cascades involving phosphoinositide 3′-kinase (PI3K), protein kinase B (Akt), GSK-3β and β-catenin in human lung epithelial BEAS-2B cells and tumor tissues (Son et al., 2012). *In vitro* studies indicated that Cd can cause tau aggregation into PHF-like filaments (Jiang et al., 2007), and *in vivo* studies showed that Cd induces the loss of basal forebrain cholinergic neurons and development of AD through activating GSK-3β and promoting Amyloid β (Aβ) protein production and tau filament formation (del Pino et al., 2015). Aberrant and sustained activation of neuronal PI3K/Akt/mTOR signaling occurred in the early stages of AD brain also mediates tau phosphorylation (Pei and Hugon, 2008). Previous reports showed that Cd induced of neuronal cell apoptosis by activation of JNK, ERK1/2, and mTOR signaling network (Chen et al., 2008, 2011) and mTOR was implicated in the pathophysiology of AD via directly and indirectly modulating tau phosphorylation (Tang et al., 2013).

L-Theanine, γ-glutamylethylamide (Fig. 1), was originally isolated from the green tea (Mu et al., 2015). L-Theanine has diverse biological activities including antioxidant properties and powerful chemopreventive capabilities in the treatment of lung cancer (Sugiyama and Sadzuka, 2003; Sugiyama et al., 2001), and it also could protect mice against alcoholic and carbon tetrachloride (CCl_4)-induced liver injury (Li et al., 2012; Pérez-Vargas et al., 2015) and enhance the antitumor activities of doxorubicin and cisplatin. Abnormally high level of extracellular glutamate is related with the pathophysiology of cerebral ischaemia and infarction. We noticed that L-theanine has been implicated to inhibit glutamate metabolism and neurotransmission within the central nervous system (Egashira et al., 2008). Several studies have shown that L-theanine has neuroprotective effect against rotenone, aluminum, 3-nitropropionic acid (3-NP), and Aβ-induced cell death (Cho et al., 2008; Kim et al., 2009; Sumathi et al., 2015; Thangarajan et al., 2014). Furthermore, L-theanine could prevent Aβ-induced mouse cognitive dysfunction (Kim et al., 2009).

Therefore, we evaluated the effects of L-theanine on Cd-induced neurotoxicity in mice. We found that L-theanine protected neurons in mouse brains against Cd-induced cell death. Mechanism study indicated that L-theanine prevented Cd-induced oxidative stress and tau protein hyperphosphorylation.

2. Materials and methods

2.1. Animals and treatments

All experimental procedures in this study were conducted in accordance with the guidelines of Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Science and Technology Department of Jiangsu Province. Adult male ICR mice (22–25 g) were obtained from Nanjing Medical University Laboratory Animal Center (Nanjing, China). Animals were maintained under standard conditions with 12-h light/dark cycles, 22 °C and 60% humidity. The food in the form of dry chow pellets and water were available *ad libitum*. Cadmium chloride ($CdCl_2$) and L-theanine were purchased from Sigma–Aldrich (Germany). L-Theanine was dissolved in saline when it was injected into mice.

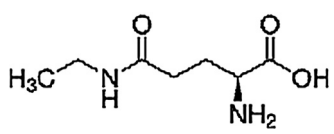

Fig. 1. Chemical structure of L-theanine.

After one week of acclimatization, mice were randomly divided into five groups (10 mice each) including control group, L-theanine alone injected group (mice were received intraperitoneal injection of 200 mg/kg L-theanine once daily for 8 weeks), $CdCl_2$ treated group (mice were orally administered with 3.75–6 mg/kg $CdCl_2$, 30 mg/L in water, for 8 weeks), Low dose L-theanine and $CdCl_2$ treated group (mice were intraperitoneally injected with 100 mg/kg/day L-theanine followed by the administration of $CdCl_2$ after 1 h for 8 weeks), and High dose L-theanine and $CdCl_2$ treated group (mice were intraperitoneally injected with 100 mg/kg/day L-theanine followed by the administration of $CdCl_2$ after 1 h for 8 weeks) (Chen et al., 2014a,b; Desai et al., 2005). At the end of treatments, the mice were deprived of food overnight, and then were anesthetized with diethyl ether. Blood was collected from the retro-orbital plexus into anticoagulant test tubes. The plasma and the brain tissue were immediately stored at −80 °C for following experiments.

2.2. Determination of Cd element contents

0.25 ml plasma sample from each animal was digested in 1 ml HNO_3 for 4 h at room temperature (Chen et al., 2014a,b), after adding 0.5 ml $HClO_4$ the plasma sample was continued to digest at 100 °C. 100 mg of brain sample was digested in 1.5 ml HNO_3 for 4 h, and then in 0.75 ml $HClO_4$ at 120 °C, until the liquid was completely evaporated (Amara et al., 2006; Zhang et al., 2012). The digested residue was dissolved in 0.7 ml HCl and diluted in pure water to 10 ml. The concentration of Cd in plasma and brain tissues were determined using inductively coupled plasma atomic emission spectroscopy (Leeman Labs, Prodigy, USA).

2.3. Detection of apoptosis

Neuronal apoptosis was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) staining (Gurel et al., 2007; Rao et al., 1998). Briefly, paraffin-embedded brain tissues were sectioned, followed by deparaffinization in xylene and rehydration in decreasing stages of ethanol. Each section was incubated with proteinase K working solution (20 μg/ml in 10 mM Tris/HCl, pH 7.4) for 15 min at room temperature and washed twice with PBS. After incubating with a reaction mix containing deoxynucleotidyl transferase and FITC–dUDP (Vazyme Biotech, Nanjing, China) for 1 h, the slides were observed under a fluorescence microscope (Nikon 80i, Japan). The intensities of fluorescence were quantified by Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). TUNEL-positive cells numbers in the investigated region are normalized with the total number of cells indicated by the DAPI stain.

2.4. Detection of ROS, MDA, GSH and antioxidant enzymes in brain tissues

Brain tissue samples were subjected to homogenization with 1:10 (w/v) PBS on ice. The homogenates were centrifuged at