Cytisine confers neuronal protection against excitotoxic injury by down-regulating GluN2B-containing NMDA receptors

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ABSTRACT

Cytisine (CYT), one of the principal bioactive components derived from the seeds of Cytisus laborinum L, has been widely used for central nervous system (CNS) diseases treatment. The present study investigated the protective effect of CYT on cultured cortical neural injury induced by N-methyl-D-aspartate (NMDA). Our data showed that CYT conferred protective effect against loss of cellular viability induced by brief exposure to 200 μM NMDA in a concentration-dependent manner. CYT significantly inhibited the neuronal apoptosis induced by NMDA exposure by reversing intracellular Ca2+ overload and balancing Bcl-2 and Bax expression levels. Furthermore, CYT significantly reversed the up-regulation of GluN2B-containing NMDA receptors by exposure to NMDA, but it did not affect the level of GluN2A-containing NMDA receptors. These findings suggest that CYT protects cortical neurons, at least partially, by inhibiting the level of GluN2B-containing NMDA receptors and regulating Bcl-2 family.

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1. Introduction

Cytisine (CYT) is mainly isolated from the seeds of Cytisus and related members of Leguminosae (Fabaceae) (Fitch et al., 2005), which has been used to treat tobacco dependence for 40 years in Eastern Europe (Etter, 2006) as a nicotinic acetylcholine receptor (nAChR) partial agonist (Fitch et al., 2005). In recent study, CYT also shows some antidepressant-like effects by blocking alpha4/beta2α2nAChRs (Mineur et al., 2007) and attenuates central nervous system (CNS) inflammation by inhibition of T-cell activities (Nizri et al., 2007). It is also a potential therapeutic drug for Parkinson's disease through prevention of the decrease of striatal dopamine tissue levels induced by 6-hydroxydopamine (Abin-Carriguery et al., 2008). But few studies focus on the action of CYT on neuroprotection.

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and plays essential roles in neural development, excitatory synaptic transmission and plasticity (Sheng and Kim, 2002). Glutamate-induced excitotoxicity in the CNS is implicated in a number of acute neurological disorders including stroke and traumatic brain injury (Arundine and Tymianski, 2004; Soundarapandian et al., 2007) as well as in chronic neurological disorders such as multiple sclerosis, Huntington’s disease, Parkinson’s disease and Alzheimer’s disease (Meldrum, 2000). Glutamate toxicity appears to be induced by excessive influx of Ca2+ into neurons through ionic channels triggered by activation of N-methyl-D-aspartate (NMDA) receptors (Gasic and Hollmann, 1992; Sattler and Tymianski, 2001). Native NMDARs are composed of GluN1, GluN2 (A, B, C, and D) and GluN3 (A and B) subunits. NMDA receptors consisting of GluN1/GluN2 heteromer seem to be the main functional units (Furukawa et al., 2005). It has been suggested that excitotoxicity is triggered by the selective activation of NMDARs containing the GluN2B subunit, which plays an important role in the pathogenesis of neurodegenerative disorders associated with glutamate excitotoxicity (Kew and Kemp, 1998). Over-activated NMDA receptors are permeable to Na+, K+, and Ca2+ ions, among which excess Ca2+ ions are linearly correlated with neuronal cell death triggered by intracellular Ca2+-dependent cascades (Lipton, 2006; Sattler and Tymianski, 2000; Simon et al., 1984). In recent years, many researchers have focused on the study of the GluN2B antagonists because of their potential cure for many nervous system diseases (Brown et al., 2011; Cho et al., 2010). In the present study, we evaluated possible neuroprotective properties of CYT against excitatory neurotoxicity mediated by NMDA in primary cultured mouse cortical neurons and elucidated its possible mechanisms. We found that CYT performed significant protective effects by down-regulating GluN2B levels and calcium overload, as well as regulating the Bcl-2 family, including Bcl-2 and Bax expression.
2. Materials and methods

2.1. Materials

Cytisine (purity > 98%) was purchased from Haoxuan Botanical Development Company Limited (Xi’an, China). NMDA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide (MTT), Ro25-6981, poly-α-lysine, trypsin, propidium iodide (PI), Hoechst 33258 and anti-β-actin antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Neurobasal medium, B27, glutamine and fetal bovine serum (FBS) were provided from Invitrogen (Carlsbad, CA, USA). Anti-MAP2, anti-GluN2A, anti-GluN2B, anti-Bax and anti-Bcl-2 antibodies were purchased from Chemicon (Temecula, CA, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from HyClone (Logan, UT, USA). All secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). M-PER Protein Extraction Buffer and enhanced chemiluminescent (ECL) solution were obtained from Pierce (Pierce, Rockford, IL). All of the other chemicals and reagents were standard commercially available biochemical quality. Stock solutions of cytisine and NMDA were made in sterile PBS, desire concentrations of both were diluted in Neurobasal medium for usage.

2.2. Primary culture of cortical neurons

Animal care and procedures were approved by the institutional animal care and use committee in full compliance with international rules and policies. Every effort was made to minimize the number of animals used and their suffering. Primary cultures of cortical neurons were prepared from the brain of E15–E16 C57BL/6 mouse embryos (obtained from the Experimental Animal Center of the Fourth Military Medical University, Xi’an, China). Briefly, dissociated cortex tissue from embryonic 15–16 days mouse was incubated with 0.125% trypsin in Ca²⁺ and Mg²⁺-free Hank’s balanced salt solution for 10 min at 37 °C. Then the cortices were washed in DMEM supplemented with 10% FBS to stop trypsin activity, and further dissociated by trituration. The single cell suspension was cultured on poly-α-lysine coated plates in neurobasal media supplemented with 2% B27, 0.5 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. It took 7 days for re-incubation, the time required for maturation of cortical neurons and half of the medium was changed every 2 days. The cells were characterized by immunohistochemistry staining for anti-MAP2 antibody, revealing that this culture procedure yielded more than 95% neurons. Neurons seeded at a density of 5 × 10⁴ cells/well in 96-well plate, 3 × 10⁵ cells/well in 24-well plate and 2 × 10⁶ cells/well in 6-well plate respectively for different treatments. Cyt was added 24 h prior to and after the addition of NMDA and was present throughout the excitotoxic injury.

2.3. Cell viability analysis

MTT assay was used to detect cell viability as described (Yang et al., 2010) with some modifications. Briefly, cortical neurons were culture in 96-well plates at 5 × 10⁴ cells/well for 7 days until matured before each treatment. For NMDA-induced injury, cells were incubated with different concentrations of NMDA (0, 50, 100, 200, 300 and 400 μM) for 30 min, then subjected to MTT assay. For CYT-mediated protection assay, the neurons were pretreated with CYT (0, 0.1, 1, and 10 μM) 24 h before subjected to NMDA (200 μM) stimulation for 30 min. For the molecule mechanisms involved, the neurons were incubated with a highly potent and selective blocker of GluN2B, Ro25-6981 (0.3 μM) for 2 h, then followed by exposure to 200 μM NMDA for 30 min. At the end of each treatment, the culture medium was replaced with fresh medium containing 0.5 mg/ml MTT for 4 h at 37 °C. After incubation, the medium was replaced by 150 μl/well dimethyl sulfoxide (DMSO) to resolve the formazan crystals. The optical density (OD) was read on a Universal Microplate Reader (EIX 800, Bio-TEK Instruments Inc., USA) at 570 nm (630 nm as a reference). The data were expressed as a percent of control value and means ± SEM of three experiments and six wells included in each group.

2.4. Hoechst/PI double staining

Cell apoptosis was determined by propidium iodide (PI) and Hoechst 33258 double fluorescent staining as described (Zhang et al., 2010) with some little changes. Neurons were cultured in 24-well plates at a density of 600 cells/mm². Neurons were pretreated with CYT (1 and 10 μM) for 24 h then subjected to excitotoxic injury with 200 μM NMDA for 30 min. One day later, the cells were stained with PI (10 μg/ml) and Hoechst 33258 (10 μg/ml) for 15 min, then the cells were fixed by 4% paraformaldehyde for 10 min. The cells were observed under a fluorescence microscope (Olympus BX61, Japan). The Hoechst and PI dye were excited at 340 and 620 nm, respectively. Hoechst 33258, a type of blue-fluorescence dye stains the condensed chromatin in apoptotic cells more brightly than normal chromatin. PI, a red-fluorescence dye, is used to discriminate late apoptotic, necrotic or dead cells which have lost membrane integrity from early apoptotic cells by dye exclusion. The staining pattern resulting from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy. Thirty random fields (total 800–1000 cells/culture) of immunostained cells were manually counted using a 20× objective.

2.5. Western blot analysis

In order to further explore the mechanisms involved in CYT-mediated neuroprotection, we examined the effects of CYT on signaling pathways related to survival by Western blot analysis as described previously (Liu et al., 2011). After each treatment, cells were rinsed twice with PBS and lysed by M-PER Protein Extraction Buffer containing 1× protease inhibitor cocktail. Cell protein was quantified by a BCA Kit and equal amounts of protein (50 μg) were separated on 10% polyacrylamide gel followed by transferred onto an Immun-Blot PVDF membrane. The membrane was blocked for 1 h with 5% non-fat milk in Tris-phosphate buffer containing 0.05% Tween 20 (TBS-T). It was further incubated overnight at 4 °C with primary antibodies including anti-GluN2A (1:1000), anti-GluN2B (1:1000), anti-Bax (1:1000) and anti-Bcl-2 (1:1000); β-actin (1:10,000) served as a loading control. After five washes for 5 min with TBS-T, membranes were further incubated with HRP-conjugated secondary antibodies for 1–2 h and followed by four TBS-T washes. The target protein signal was detected and digitalized using ECL and Image J program.

2.6. Calcium imaging

Calcium imaging was performed as described earlier (Shen et al., 2008). Neurons were cultured in 3.5 mm plates which were made specially for laser scanning microscope at a density of 3 × 10⁴ per plate. Cultured cells were washed twice using Mg²⁺-free extracellular solution (ECS) containing (in mM): NaCl 140, KCl 3, CaCl₂ 2, HEPES 10, glucose 10. The pH was adjusted to 7.2–7.3 with NaOH and osmotic pressure adjusted to 310 ± 5 with sucrose. The neurons were incubated with 2.5 μM fluo-3/AM at 37 °C for 30 min, followed by twice washes, then recovered in the original culture medium for an additional 30 min. The dye-loaded cells were measured for fluorescence using a confocal laser scanning microscope.
Then, picked and scanned expressed viability determined (Olympus). Prior to NMDA application, the dye-loaded cells were scanned for about 1 min to obtain a basal level of intracellular Ca$^{2+}$. Then, 200 μM NMDA was applied to the cultures, and an equal amount of ECS was added as a placebo. CYT was added 24 h before the experiments and existed in the whole experiment process. The change of Ca$^{2+}$ concentration was estimated by the fluorescence ratio of the fluo-3/AM loaded neurons for another 4 min. The results expressed as the changes from the basal level, and five cells were picked out randomly for analysis.

Fig. 1. Cytisine promoted neurons viability upon NMDA injury. (A) Concentration-dependent cytotoxic effects of NMDA on the cell viability of cortical neurons. Primary cultures of mouse neurons were treated with NMDA for 30 min and cell viability was determined by MTT method. (B) Time-dependent cytotoxic effects of NMDA on the cell viability of cortical neurons. Primary cultures of mouse neurons were treated with 200 μM NMDA for 0, 10, 20, 30 and 40 min and cell viability was determined by MTT method. (C) Effects of CYT and Ro25-6981 on the cell viability of cortical neurons after exposure to NMDA. Primary cultures of mouse neurons were treated with CYT at different concentration for 24 h, or with 0.3 μM Ro25-6981 (GluN2B antagonist) for 2 h, followed by exposure to 200 μM NMDA for 30 min and cell viability was determined by MTT method. *P < 0.05, **P < 0.01 versus control group and #P < 0.05, ##P < 0.01 versus 200 μM NMDA alone.

2.7. Data analysis

Data were presented as means ± SEM for three separate experiments (each in triplicate). The data were evaluated statistically by analysis of variance followed by the Tukey’s test for paired observations. Significance was considered to be less than 0.05. To account for any donor-specific differences, all experiments were performed from three donors at least.

3. Results

3.1. Cytisine promoted neuron viability upon NMDA injury

NMDA has been evidenced to be involved in the pathogenesis of neurodegenerative disorders associated with glutamate...
excitotoxicity. In order to determine whether CYT confers neuroprotection, we evaluated the effects of CYT on NMDA induction in cultured mouse cortical neurons firstly. The neurons were exposed to an increasing concentration of NMDA (0, 50, 100, 200, 300, 400 μM) for 30 min. As expected, our results indicated NMDA decreased cell viability in a concentration-dependent manner (Fig. 1A) and time-dependent manner (Fig. 1B) as measured by MTT assay. An exposure to 200 μM NMDA for 30 min was used in subsequent experiments since cell insult was significant in this paradigm (cell viability in NMDA treated: 62.2 ± 2.2%, P < 0.01 versus control alone). Pretreatment with CYT at 10 μM for 24 h showed effective neuroprotection against NMDA injury as illustrated in Fig. 1C (cell viability in 10 μM CYT treated: 89.4 ± 5.0%, P < 0.01 versus NMDA alone). No significant changes in the viability of cortical neurons were found in 10 μM CYT alone treated group compared with the control, indicating that CYT itself exerted no toxicity on the cultured neurons (Fig. 1C).

To further determine the neuroprotective effects of CYT, we carried out Hoechst 33258 and PI double-staining. Data demonstrated that 8.2 ± 2.7% cells in control group underwent apoptosis, while 34.0 ± 2.57% underwent in NMDA insult group (P < 0.01 versus control, Fig. 2A and B). However, CYT (1 μM and 10 μM) significantly attenuated excitotoxicity of NMDA on cortical neurons. The percentage of cells in 1 μM CYT group undergoing apoptosis was decreased to 23.9 ± 2.1% (P < 0.01 versus NMDA alone; Fig. 2A and B), and in 10 μM CYT group the percentage of apoptotic cells was decreased to 12.3 ± 3.1% (P < 0.01 versus NMDA alone; Fig. 2A and B). These data suggested that CYT protected neurons from excitotoxicity induced by NMDA.

3.2. Cytisine affected Bcl-2 and Bax levels in neurons

The possible relationship between NMDA-induced cell death and potential intracellular mediators was assessed in experiments that measured Bax and Bcl-2 levels. Western blot analysis showed that Bcl-2 and Bax were both expressed in non-injured cortical neurons, and CYT (10 μM) treatment alone did not alter the level of these proteins (Fig. 3A). It was significantly changed in the total amount of pro-apoptotic protein Bax (148.5 ± 13.3% of control, P < 0.05; Fig. 3A and B) and anti-apoptotic protein Bcl-2 (22.2 ± 6.5% of control, P < 0.01; Fig. 3A and C) in cultured cortical neurons after exposure to NMDA. However, 10 μM CYT showed an anti-apoptotic activity by increasing Bcl-2 (59.8 ± 4.7% of control, P < 0.01 versus NMDA alone; Fig. 3A and C) and inhibiting Bax level (80.79 ± 12.6% of control, P < 0.05 versus NMDA alone; Fig. 3A and B). Accordingly, the ratio of Bax/Bcl-2 was increased by NMDA stimulation (6.68-fold of control, P < 0.01 versus control; Fig. 3D) and reversed by CYT pretreatment (1.35-fold of control, P < 0.01 versus NMDA alone; Fig. 3D). The effects of CYT on the Bax/Bcl-2 ratio may constitute an important element responsible for neuroprotection.

3.3. Cytisine attenuated the up-regulation of calcium in neurons induced by NMDA

NMDARs activation increases cytoplasmic calcium concentration in the cultured neurons (MacDermott et al., 1986), and Ca^{2+} overload triggers multiple intracellular catabolic processes followed by an irreversible death of neuronal cells in the brain (Liu et al., 2004). Next, we focused on the effects of CYT on the Ca^{2+} overload. The fluorescence intensity can be regarded as an indicator of cytoplasmic Ca^{2+} concentration (Matsumoto et al., 2004). We found the Ca^{2+} concentration in cultured neurons was stable during detection time (Fig. 4A and B), and NMDA (200 μM) evoked a fast elevation of Ca^{2+} concentration in cultured neurons (Fig. 4A and C). A slow reduction was observed in the next 4 min, after which the Ca^{2+} concentration stayed at a relatively higher stage. 10 μM CYT attenuated the amplitude and speed of the elevation in Ca^{2+} concentration (Fig. 4A and C).
3.4. Cytisine affected levels of GluN2A- and GluN2B-containing NMDARs

It has been suggested that GluN2A- and GluN2B-containing NMDARs are associated with different intracellular cascades and participate in different functions in neuronal cell survival or death (Liu et al., 2007). Activation of either synaptic or extra synaptic GluN2B-containing NMDARs results in excitotoxicity as increased neuronal apoptosis (Brown et al., 2011; Cho et al., 2010). In contrast, activation of either synaptic or extra synaptic GluN2A-containing NMDARs promotes neuronal survival and exerts neuroprotection against either NMDARs-mediated or non-NMDARs-mediated neuronal damage (Liu et al., 2007). To further examine CYT effects on the expression of NMDAR subtypes, we performed Western blot analysis on the level of GluN2B-containing NMDARs of neurons after exposure to NMDA. The results showed that GluN2B subtype level was notably increased in cultured cortical neurons after exposure to NMDA (235.3 ± 17.51% of control, P < 0.05; Fig. 5A and B), whereas the GluN2A subtype level was not changed (89.8 ± 17.5% of control, P > 0.05; Fig. 5A and C). CYT blocked up-regulation of GluN2B subtype upon NMDA stimuli markedly as showed in Fig. 4A and B (126.0 ± 14.3%; P < 0.05 versus NMDA alone). However, CYT had no effects on the GluN2A subtype level (103.0 ± 12.9% of control; P > 0.05 versus NMDA alone; Fig. 5A and C). CYT treatment alone did not alter the level of these proteins (Fig. 5A–C). Additionally, CYT may protect neurons from NMDA cytotoxicity through down-regulation of GluN2B subtype level rather than GluN2A subunit.

4. Discussion

The importance of the present findings is that cytisine, one of the active ingredients of Cytisus, significantly attenuates the neuronal injury induced by NMDA stimuli as shown by the results
Glutamate activates three classes of ionophore-linked postsynaptic receptors, including NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors (Collingridge and Lester, 1989). It is clear that NMDARs are involved in synaptic plasticity, learning, memory and neurological diseases (Badanich et al., 2011; Cao et al., 2007; Chang et al., 2009; Cui et al., 2011; D’Mello et al., 2011; Day et al., 2011). The pathologic activation of NMDARs contributes to neuronal death after acute excitotoxic trauma such as brain ischemia (Arundine and Tymianski, 2004; Guo et al., 2011). NMDA receptor toxicity is dependent on extracellular Ca\(^{2+}\), and reflects a large amount of Ca\(^{2+}\) influx directly through the receptor-gated ion channels (Lipton, 2006; Simon et al., 1984). NMDARs assemblies differently due to the diversity and GluN1/GluN2 heteromer seems to be the main functional unit (Furukawa et al., 2005). Both GluN1/GluN2A and GluN1/GluN2B subunit assemblies have different developmental, anatomical and functional profiles that allow them to play different roles in normal and pathological situations (Erreger et al., 2007). NMDARs consisted of GluN2B subunit promote neuron protection, whereas GluN2B-containing NMDARs mediate excitotoxicity (Brown et al., 2011; Cho et al., 2010). Therefore, it is likely that specific antagonists selective for a particular NMDAR subunit are quite beneficial for the future development of therapeutic drugs useful for neurodegenerative diseases relevant to glutamate excitotoxicity. Activation and opening of NMDAR channels causes an influx of cations and opening of Ca\(^{2+}\) channels (Birnbaumer et al., 1994). The subsequent influx of Ca\(^{2+}\) causes further glutamate release and initiates the process of neuronal cell loss. Ca\(^{2+}\) overload triggers multiple intracellular catabolic processes and several downstream lethal reactions including nitrosative stress, oxidative stress and mitochondrial dysfunction (Meldrum, 2000). In this study, the elevation of Ca\(^{2+}\) stimulated by NMDA is inhibited by CYT in a concentration-dependent manner to support neuroprotection.

It is clear that glutamate evoked different intracellular cytotoxic signals, among these, Bcl-2 family proteins play critical roles in apoptotic cell death (Antonsson, 2004). Bcl-2, an antiapoptotic protein, is predominantly present in mitochondria and inhibits various agents-mediated apoptosis (Itoh et al., 1993). It has been showed that Bcl-2 inhibits cell death by suppressing oxymediated membrane damage, stabilizing mitochondrial membrane potential and preventing cytochrome c release (Bruce-Keller et al., 1998). In contrast to Bcl-2, Bax is pro-apoptotic and is shown to be at least partly responsible for excitotoxic apoptotic cell death mediated by NMDAR in neurons (Gu et al., 2009; McInnis et al., 2002). In this study, CYT significantly suppressed Bax level induced by NMDA and increased Bcl-2 level to serve as antiapoptotic elements.

Taken together, the present study indicates that CYT might protect cortical neurons through inhibiting apoptosis evoked by over-activation of GluN2B-containing NMDARs. These findings have partially revealed the molecular mechanisms underlying neuroprotection of CYT. However, we could not exclude the possibility that CYT may undergo neuroprotective activities through other pathways. Other supportive experiments need to be performed for further investigation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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