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Neuroprotective effects of curculigoside against NMDA-induced neuronal excitoxicity in vitro

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ABSTRACT

Glutamate is an important excitatory neurotransmitter in the central nervous system. Excessive accumulation of glutamate can cause excitotoxicity, which plays a key role in spinal cord injury, traumatic brain injury, stroke, and neurodegenerative diseases. Curculigoside (CCGS) is a major bioactive compound isolated from the rhizome of *Curculigo orchioides* Gaertn. CCGS has an extensive biological effect and has been used in Traditional Chinese Medicine. However, little is known about the neuroprotective effects of CCGS on glutamate-induced excitotoxicity. This study aims to evaluate the neuroprotective effects of CCGS in cultured cortical neurons. The results indicated that treatment with 1 and 10 μM CCGS evidently prevented *N*-methyl-D-aspartate (NMDA)-induced neuronal cell loss and reduced the number of apoptotic and necrotic cells in a time- and concentration-dependent manner. The neuroprotective effects of CCGS are related to down regulating the apoptotic protein levels and reducing the production of intracellular reactive oxygen species in cultured cortical neurons. These findings give a new insight into the development of natural anti-excitotoxicity agents.

1. Introduction

Excitotoxicity plays a key role in spinal cord injury, traumatic brain injury, stroke, and neurodegenerative diseases of the central nervous system (CNS), such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and alcohol withdrawal (Doble, 1999; Ikonomidou and Turski, 1995). Excitotoxicity is the pathological process by which nerve cells are damaged and killed by excessive glutamate stimulation. Glutamate is the principal excitatory neurotransmitter in mammalian CNS and plays a key role in brain metabolism; it is also involved in most aspects of normal brain functions including cognition, memory, and learning (Danbolt, 2001). However, glutamate is also a potent neurotoxin. High glutamate concentration is harmful to the brain, and glutamate antagonists can inhibit this neurotoxicity (Olney, 1969). The overactivation of glutamate receptors can trigger high calcium ion (Ca^{2+}) influx, which activates a number of enzymes that damage cell structures such as the cell membrane, cytoskeleton components, and DNA. This Ca^{2+} influx is thought to contribute to Ca^{2+}-mediated excitotoxic neuronal cell death in the above-mentioned disease processes (Brorson et al., 1994; Manev et al., 1989).

Curculigo orchioides Gaertn is a small herb widely distributed in China, India, Malaya, Japan, and Australia. In China, it is a rhizome known as "Xian Mao" and is a common kidney-Yang-reinforcing and anti-aging traditional Chinese medicine (Wu et al., 2005). Curculigoside (CCGS) or β-D-glucopyranoside,2-{[(2,6-dimethoxybenzoyl)oxy]methyl}-4-hydroxyphenyl (Fig 1) is a major compound isolated from the rhizome of *C. orchioides* Gaertn. CCGS has an extensive biological effect. It promotes macrophage phagocytosis and attenuates hydrogen peroxide (H_2O_2)-induced human umbilical vein endothelial cell injury (Wang et al., 2010). Other researchers revealed that CCGS exhibits an antiosteoporotic activity (Jiao et al., 2009; Wang et al., 2012) and affects bone formation and fracture healing (Ma et al., 2011). Jiang et al. reported that CCGS attenuates cerebral ischemic injury in rats in vitro and in vivo (Jiang et al., 2011). However, little is known about the effects of CCGS on glutamate-induced neuronal apoptosis. This study aims to investigate the effects of CCGS on neuronal excitotoxicity.

2. Materials and methods

2.1. Materials

CCGS was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Anti-pro-caspase-3, anti-bax and anti-Bcl-2 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum,

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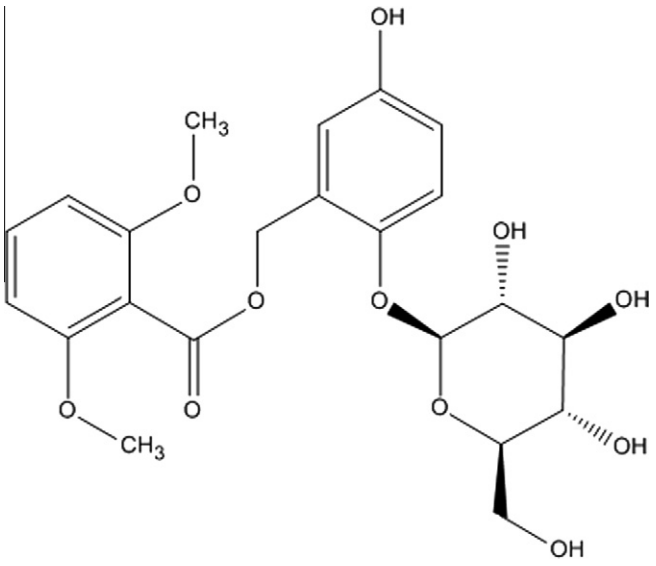

Fig. 1. Chemical structures of curculigoside (CCGS).

Neurobasal medium, and B27 were obtained from Gibco Life Technologies (Grand Island, NY). All of the chemicals and reagents used are commercially available and of standard biochemical quality.

2.2. Primary mouse cortical neuronal culture

The Animal Care and Use Committee of the Fourth Military Medical University approved all animal protocols. Cultured prefrontal cortex neurons were derived from E18 C57Bl/6 mice as Wang et al. described (Wang et al., 2008). Briefly, the prefrontal cortex was dissected, minced, and trypsinized for 15 min using 0.125% trypsin (Invitrogen, Carlsbad, USA). Cells were then seeded onto 96-well plates, 24-well plates, 6-well plates, or 100 mm dishes. All plates were pre-coated with 50 μg/ml poly-D-lysine (Sigma) and grown in Neurobasal medium (Invitrogen) supplemented with B27 and 2 mM glutamine (Invitrogen). In B27/Neurobasal medium, glial growth was reduced to less than 0.5% as assessed by immunocytochemistry for glial fibrillary acidic protein (GFAP). The vast majority of cultured cells were immunoreactive for neuron-specific enolase (Brewer et al., 1993). The cultures were incubated at 37 °C in 95% air/5% carbon dioxide with 95% humidity. Cultures were used for experiments on the 10th day in vitro (DIV 10). The neurons were briefly rinsed with phosphate-buffered saline (PBS) and added new Neurobasal medium without B27, and then treated with CCGS for different times. NMDA (200 μM) and glycine (10 μM) were added to the medium with CCGS for 30 min. The cells were washed twice and returned to the original culture medium for another 24 h.

2.3. Cell viability analysis

The MTT assay was used to detect cell viability as Shen et al. described (Shen et al., 2008). Neurons were cultured in 96-well plates at a density of 8×10^4 cells per well. The substrate MTT was dissolved in DMEM medium and added to each well at a final concentration of 0.5 mg/ml and then incubated at 37 °C for 4 h .Then the medium was then replaced by 150 μl dimethyl sulfoxide (DMSO) to dissolve the formazan product. The optical density (OD) was read on a Universal Microplate Reader (Elx 800, Bio-TEK instruments Inc., USA) at 570 nm (using 630 nm as a reference). Cell viability was presented as a percentage of the absorbance of untreated cultures. All data are expressed as mean ± SEM of three independent experiments and each mean included data from six wells.

2.4. Hoechst 33258 and PI double staining

Cell death was determined by propidium iodide (PI, Sigma) and Hoechst 33258 (Sigma) double fluorescent staining as Shen et al. described (Shen et al., 2008). Neurons were cultured in 24-well plates at a density of 600 cells/mm^2. Twenty-four hours after NMDA treatment, the cells were stained with PI (10 μg/ml) and Hoechst 33258 (10 μg/ml) for 15 min, and then fixed in 4% paraformaldehyde for 10 min. Hoechst 33258 is excited by UV light at around 350 nm and emits blue fluorescence light at 461 nm. Hoechst 33258 is often used to distinguish the compact chromatin of apoptotic nuclei from that of normal cells (Gonzalez-Juanatey et al., 2004). Propidium iodide, a red-fluorescence dye (excited at 620 nm), is only permeant to dead cells. Staining was imaged and analyzed using Olympus Fluoview FV100 (Olympus, Japan). To assess apoptotic nuclei and dead/dying neurons, three visual fields were randomly selected from each well.

2.5. Flow cytometric analysis

Flow cytometric analysis of apoptosis was performed as Behbahani et al. described (Behbahani et al., 2005). Briefly, apoptosis and cell viability were measured by using annexin V-FITC (Assay Designs, Inc., USA) and PI double staining. Primary cultures were harvested and washed with PBS. The cells were diluted in 100 μl of 1× annexin-binding buffer and incubated darkly with annexin V-FITC and PI for 15 min at room temperature. Afterwards, 400 μl of 1× binding buffer was added. The stained cells were analyzed immediately by flow cytometry. Annexin V-FITC and PI were used to identify populations of viable cells (annexin V^-PI^-), early apoptotic cells (annexin V^+PI^-), late apoptotic cells (annexin V^+PI^+), and necrotic cells (annexin V^-PI^+). Cell fluorescence was analyzed on a FACScan flow cytometer (Becton–Dickinson, San Jose, CA) and analyzed using CELLQuest software (Becton–Dickinson, San Jose, CA).

2.6. Western blot analysis

Western blot analysis was performed as Chen et al. described (Chen et al., 2008). Equal amounts of protein (50 μg/lane) from the cultures were separated and electrotransferred onto PDVF membranes (Invitrogen). Membranes were probed with anti-pro-caspase-3 (1:1000), anti-Bcl-2 (1:500), anti-Bax (1:500), and with β-actin (1:10000) as a loading control. For data quantitation, band intensities were expressed relative to the loading control (β-actin). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG depending on the primary antibody), and bands were visualized using an enhanced chemiluminescence (ECL) system (Perkin Elmer).

2.7. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS was monitored using a 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe. DCFH cannot cross the cell membrane. DCFH-DA is nonfluorescent; however, the probe can be easily loaded into the cell. It freely crosses the cell membrane into the cell and is hydrolyzed to DCFH by intracellular esterases. ROS oxidize DCFH to generate the fluorescent dichlorofluorescin (DCF). The detection of DCF fluorescence indicates the level of intracellular ROS. At the end of treatment, cells were incubated with 10 mM DCFH-DA at 37 °C for 30 min and then washed twice with phosphate-buffered saline. Finally, the fluorescence intensity of DCF was measured by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.8. Data analysis

Results are expressed as means ± SEM. Statistical comparisons were performed using one way ANOVA using the Tukey's test for post hoc comparisons. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. CCGS attenuated NMDA-induced cell loss in cultured cortical neurons

To test for the neuroprotective effects of CCGS, cultured cortical neurons (DIV 10) were treated with CCGS for different times and treated with NMDA (200 μM) and glycine (10 μM) for another 30 min. The cells were returned to the original culture medium for 24 h (Fig 2a). As determined through MTT assay, exposure to NMDA decreased cell viability. First, we found that CCGS (1 and 10 μM) attenuated the cell loss by the NMDA exposure in a time-dependent manner (Fig. 2b and c). Pretreatment or co-treatment of CCGS with NMDA could attenuate the cell loss; however, post-treatment of CCGS 1 h after NMDA exposure did not change the cell viability (Fig. 2b and c). Next, we found that CCGS significantly increased the cell viability of the NMDA-exposed neurons in a concentration-dependent manner (Fig. 2d). This protection reached to the peak at the concentration of 100 μM, suggesting that 1 and 10 μM CCGS could produce the best neuroprotective effects in cultured cortical neurons. Therefore, 1 and 10 μM CCGS were used in the following experiments, although CCGS (1, 10, and 100 μM) alone showed no effect on cell viability (Fig. 2e).

3.2. CCGS protected neurons from NMDA-induced apoptosis in cultured cortical neurons

To confirm the neuroprotective effects of CCGS against NMDA-induced cell loss in cultured cortical neurons, Hoechst 33258 and