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Phenylethanoid glycosides from *Cistanches salsa* inhibit apoptosis induced by 1-methyl-4-phenylpyridinium ion in neurons

Xue-Fei Tian, Xiao-Ping Pu*

Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Science, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100083, PR China

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Abstract

In our study we investigated the neuroprotective effects of phenylethanoid glycosides (PhGs) from *Cistanches salsa* on 1-methyl-4-phenylpyridinium ion (MPP^+)-induced apoptosis in cerebellar granule neurons (CGNs). CGNs were treated with 100 μM MPP^+ for 24 h to induce apoptosis, simultaneously CGNs were incubated with PhGs at 10, 20 and 40 μg/ml, respectively. In addition CGNs were pretreated with PhGs at 20 μg/ml for 6, 12, 24 h, respectively, and then treated with 100 μM MPP^+ for 24 h.

3-(4,5-Dimethylthiazol-2-ylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that the treatment of CGNs with PhGs inhibited the decrease of cell viability induced by MPP^+. The activation of caspase-3 and caspase-8 was induced by MPP^+ in apoptosis. The caspase-3 and caspase-8 fluorogenic assays showed that the treatments of CGNs with PhGs efficiently suppressed the activation of caspase-3 and caspase-8 induced by MPP^+. It is concluded that PhGs can prevent the MPP^+-induced apoptosis in CGNs and exert its anti-apoptosis effect by inhibiting caspase-3 and caspase-8 activities.

Keywords: *Cistanches salsa*; Phenylethanoid glycosides (PhGs); Apoptosis; Caspases; Parkinson's disease (PD)

1. Introduction

Inappropriate apoptosis has been suggested to be involved in neuronal death which occurs in various human neurodegenerative disorders, including Parkinson's disease (PD) (Nijhawan et al., 2000; Sastry and Subba, 2000). Moreover, several studies show that apoptosis occurs after transient focal or global cerebral ischemia in rodents (MacManus et al., 1993; Li et al., 1995). Primary cultures of cerebellar granule neurons (CGNs) have been proposed as a suitable in vitro model for studying the mechanisms of MPP^+-induced neuronal apoptosis (Du et al., 1997; Kalivendi et al., 2003).

Cistanches salsa (C.A. Mey) G. Beck is a parasitic plant native to the northwest of China. The stem of this plant is an important traditional Chinese medicine and is used for the treatment of kidney deficiency and neurasthenia. The major active constituents of this herb are phenylethanoid glycosides (PhGs) (Jimenez and Riguera, 1994). Many PhGs were shown to have a wide range of biological properties, including antioxidant and antitumor effects (Andary, 1993). Acteoside of PhGs from *Cistanches salsa* inhibits apoptosis induced by MPP^+ in CGNs (Pu et al., 2003). However, it is not clear whether PhGs have neuroprotective effects and affect MPP^+-induced apoptosis/activation of caspases or not. In our study we investigated the effects of PhGs from *Cistanches salsa* on MPP^+-induced cell apoptosis in CGNs, and found PhGs significantly improved cell viability and inhibited MPP^+-induced apoptosis in rat CGNs. PhGs efficiently suppressed the activation of caspase-3 and caspase-8 induced by MPP^+.

* Corresponding author. Tel.: +86 10 82802431; fax: +86 10 82802431.
E-mail address: pxp123@bjmu.edu.cn (X.-P. Pu).

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2. Materials and methods

2.1. Materials

The stems of *Cistanches salsa* (C.A. Mey) G. Beck were collected in Yutian County, Xinjiang Autonomous Region, P.R. China and identified by Dr. Chen Hubiao, Department of Botany, School of Pharmaceutical Sciences, Peking University, and the voucher specimen is deposited in the Herbarium of School of Pharmaceutical Sciences, Peking University (number: 200211). PhGs from the stems of *Cistanches salsa* were extracted by the method of Lei et al. (2001). They were identified as 40% echinacoside and 10% acteoside and 40% other 10 trace of PhGs on the basis of chemical evidence and spectral data.

Minimum essential medium (MEM) and fetal calf serum were purchased from Gibco BRL; poly-L-lysine, MTT and MPP^+ were purchased from Sigma (St. Louis, MO, USA).

Epidermal growth factor (EGF) was purchased from Pepre (London, UK). Caspase-3 assay kit, rhodamine 110-based caspase-8 substrate and caspase-8 inhibitor were obtained from Molecular Probes (Eugene, OR, USA). Five-day-old Wistar rats were obtained from Department of Laboratorial Animals, Peking University. All other reagents were of analytical grade.

2.2. CGNs culture and treatments

Primary cultures of CGNs were prepared from 5-day-old Wistar rats according to the procedure as described previously (Levi et al., 1984) and the purity of CGNs was more than 95%. Dissociated cells were maintained in MEM, supplemented with 10% inactivated fetal calf serum, 25 mM KCl and 2 mM L-glutamine in a humidified incubator aerated with 95% air and 5% CO_2 at 37 °C. CGNs 1×10^6/ml were cultured in 96-well plastic plates (MTT assay) or 25-cm culture flasks (caspases assay) coated by poly-L-lysine in advance. 1-β-D-Arabinofuranosylcytosine (10 μM) was added to the culture medium 24 h after culture to prevent replication of non-neuronal cells. All experiments were carried out with fully differentiated neurons (5 days in culture).

2.3. Analysis of cell viability

The cell viability was determined using a modified MTT assay as described previously (Yamamoto et al., 2000). The CGNs were grown for 5 days, and then the culture medium was replaced by medium containing various concentrations of PhGs (10, 20 and 40 μg/ml), EGF (100 ng/ml) or 100 μM MPP^+, besides cells were treated with 20 and 40 μg/ml PhGs alone for 24 h. In addition, to investigate PhGs effects in various incubation time, CGNs were pretreated with PhGs at 20 μg/ml for 6, 12 or 24 h, respectively, and then treated with 100 μM MPP^+ for 24 h at the same time point. The growth condition and cultured-time of CGNs in all groups were the same. After incubation for up to 24 h, MTT solution (5 mg/ml) was added to the 96-well plates and the cells were allowed to incubate for 4 h at 37 °C After the medium had been removed, the cells and dye crystals were solubilized by 200 μl of dimethylsulfoxide (DMSO), and the absorption was measured at 570 nm (540 nm as a reference) with a model 550-microplate reader (Bio-Rad).

2.4. Assay for caspase-3 activity

The activation of caspase-3 was determined with the caspase-3 activity assay kit as described method. Briefly, CGNs were incubated with PhGs (10, 20 and 40 μg/ml) or 100 μM MPP^+ for 24 h, besides cells were treated with 40 μg/ml PhGs alone for 24 h. In evaluating PhGs effects in various incubation time, the cell treatments were the same as those in MTT assay. Cells were collected (2 millions per sample), washed twice with phosphate-buffered saline (pH 7.4), and resuspended in 50 μl pre-cooled lysis buffer comprising 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% tritonX-100, 1 mM DTT and 1 mM EDTA. The cells were allowed to swell for 20 min on ice. After incubation, the cellular lysates were centrifuged at 10,000 rpm for 15 min at 4 °C to precipitate the cellular debris.

Fifty microliters cell lysate was incubated with the same volume of the reaction buffer consisting of 20 mM piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES, pH 7.4), 4 mM ethylene diamine tetraacetic acid (EDTA), 0.2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and 50 μM rhodamine 110 bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110), and was incubated at 37 °C for 30 min in the dark. As an additional control, 10 μM Ac-DEVD-CHO inhibitor solution was added to selected samples and incubated at 37 °C for 10 min. Other samples (without inhibitor) were stored on ice during this time. The intensity of fluorescence of Z-DEVD-R110 substrate was then measured in a fluorescence microplate reader using excitation at 485 ± 10 nm and emission detection at 535 ± 12 nm.

2.5. Measurement of caspase-8 activity

The caspase-8 substrate, rhodamine 110 bis-(*N*-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide) (Z-IETD-R110) was solved in DMSO and was diluted into 50 μM just prior to initiating the reaction by adding the enzyme preparation or the cell lysate. The cells were treated, and lysed prior to assaying for caspase-8 activity using lysis buffer as described above. The assays with the caspase-8 substrate were typically performed at 37 °C. in 10 mM PIPES buffer (pH 7.4), 2 mM EDTA and 0.1% CHAPS, using the same methods as the measurement of caspase-3 activity. Caspase-8 inhibitor, Z-I-E(OMe)-T-D(OMe)-FMK, was reconstituted using DMSO and diluted into 50 μM with 10% fetal calf serum MEM culture. The intensity of fluorescence of Z-IETD-R110 substrate was then measured in a fluorescence microplate reader using excitation at 498 ± 10 nm and