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Involvement of IGF-I receptor and estrogen receptor pathways in the protective effects of ginsenoside Rg1 against $A\beta_{25-35}$-induced toxicity in PC12 cells

Wen-Fang Chen [a,*], Li-Ping Zhou [a], Lei Chen [a], Lin Wu [a], Quan-Gui Gao [a,b], Man-Sau Wong [b]

[a] Department of Physiology, Shandong Provincial Key Laboratory of Pathogenesis and Prevention of Neurological Disorders and State Key Disciplines: Physiology, Medical College of Qingdao University, Qingdao 266071, China
[b] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China

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ABSTRACT

Ginsenoside Rg1 is the main pharmacologically active compound of ginsenosides and has demonstrated pharmacological effects in the cardiovascular system, central nervous system and immune system. The involvement of insulin-like growth factor-I receptor (IGF-IR)-dependent pathway and estrogen receptor (ER)-dependent pathway in the biological effect of ginsenoside Rg1 have been demonstrated in our previous study. The present study tested the hypothesis that the protective effects of Rg1 against $A\beta_{25-35}$-induced toxicity involved activation of the IGF-IR and ER signaling pathways in PC12 cells. Treatment with $A\beta_{25-35}$ decreased the cell viability in a dose-dependent manner in PC12 cells. Rg1 pretreatment resulted in an enhancement of survival and the maximum protection occurred at the concentration of 1 μM. Co-treatment with IGF-IR antagonist JB-1 or ER antagonist ICI182,780 could completely block the protective effect of Rg1. The decreased Bcl-2 mRNA expression induced by $A\beta_{25-35}$ could be restored by Rg1 pretreatment. Rg1 pretreatment could also restore the decreased mitochondrial membrane potential induced by $A\beta_{25-35}$ and these effects could be completely blocked by JB-1 or ICI182,780. In addition, Rg1 treatment alone could significantly increase the phosphorylation level of MEK and ERK in a time-dependent manner and the functional transactivation of ERα in PC12 cells. The functional transactivation of ERα by Rg1 could be completely blocked by JB-1 or ICI182,780. Taken together, our results suggest that IGF-IR and ER signaling pathways might be involved in the protective effect of Rg1 against $A\beta_{25-35}$-induced toxicity in PC12 cells.

1. Introduction

Alzheimer's disease (AD) is one of the most common age-related neurodegenerative diseases. The major pathological hallmarks of AD include extracellular amyloid plaques and intracellular neurofibrillary tangles in hippocampal and cerebral cortical regions. Epidemiological data show that age-related loss of estrogen has been related to an increased risk of AD. Hormone therapies have the potential to combat AD pathogenesis (Carroll and Rosario, 2012; Garcia-Segura et al., 2001; Wharton et al., 2011). Experimental evidence indicates that estrogens can promote the survival of neurons and inhibit low-order Aβ oligomer formation (Morinaga et al., 2011). Despite indications of benefits, the potential protection of hormone therapy against AD remains controversial. Many women turn to phytoestrogen as an alternative to hormone replacement therapy because of the side effects of estrogen, such as increased risk of breast and endometrial cancer and irregular bleeding (Reeves et al., 2006).

Ginseng is a famous Chinese herbal medicine and has been used as a medicine for over two thousand years. Ginsenosides are triterpenes saponins considered to be the main bioactive ingredients of ginseng. Studies have shown that ginsenosides possess a variety of beneficial effects on human health, including anti-inflammatory, antioxidant, anticancer, antistress and immunomodulatory effect (Qi et al., 2010; Christensen, 2009). Ginsenoside Rg1 is the main pharmacologically active compound of ginsenosides (Fig. 1). Many studies have shown the neuroprotective effects of ginsenoside Rg1 both in *in vivo* and *in vitro*. In *in vitro* studies, ginsenoside Rg1 could protect against β-amyloid peptide (Aβ)-induced neurotoxicity (Wang and Du, 2009; Gong et al., 2011). Rg1 could promote non-amyloidogenic cleavage of beta-amyloid precursor protein (APP) via estrogen receptor (ER) (Shi et al., 2011). *In vivo* studies have demonstrated that ginsenoside Rg1 can protect against the toxicity of Aβ (Wang et al., 2001). Long-term consumption of Rg1 could increase PKA/CREB activity and brain derived neurotrophic factor content in the hippocampus in senescence-accelerated mouse prone 8 (SAMP8) mice (Shi et al., 2010). However, the exact

* Corresponding author. Address: Department of Physiology, Medical College of Qingdao University, No. 308 Ningxia Road, 402 Boya Building, Qingdao 266071, China. Tel.: +86 532 83780051; fax: +86 532 83780136.
E-mail address: marychhk@yahoo.com (W.-F. Chen).

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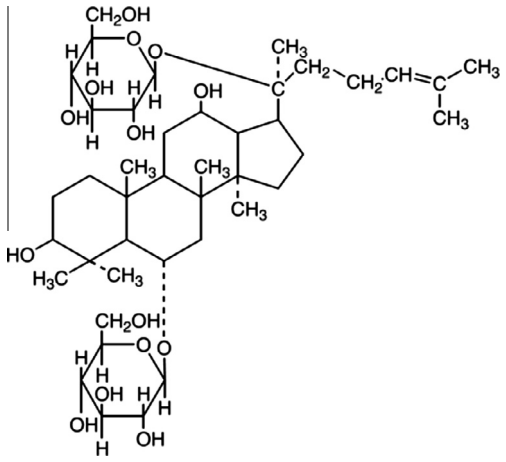

Fig. 1. Chemical structure of ginsenoside Rg1.

mechanism of the neuroprotective effect of Rg1 against β-amyloid peptide needs further study.

Our previous study has demonstrated that ginsenoside Rg1 stimulated the proliferation of human breast cancer MCF-7 cells as well as activated estrogen receptor response element (ERE)-dependent luciferase activities in Hela cells without direct binding to estrogen receptor (ER). The ER antagonist ICI182, 780 could completely block the cell proliferation and ERE luciferase activities in MCF-7 cells, indicating the involvement of ligand-independent activation of ERα pathway in the actions of Rg1 (Chan et al., 2002; Lau et al., 2008). Our study also showed that Rg1 could activate the insulin like growth factor-1 receptor (IGF-IR) signaling pathway by enhancing tyrosine phosphorylation of insulin receptor substrate-1 and mitogen-activated protein kinase kinase (MEK), which subsequently activate phosphrylation of ERα Ser118 residue in MCF-7 cells (Chen et al., 2006). In addition, pretreatment with Rg1 significantly attenuated the loss of SNpc neurons in 6-OHDA model of nigrostriatal injury. Blockage of IGF-IR by intracerebroventricular JB-1 attenuated the neuroprotective effects of Rg1 (Xu et al., 2009). In our *in vitro* study, pretreatment with Rg1 could attenuate 6-OHDA-induced cell death, cell cycle arrest and the decrease in mitochondrial membrane potential in SK-N-SH cells. These effects could be abolished by JB-1 or ICI182,780 (Gao et al., 2009). Our results suggested that IGF-IR and ER signaling pathways were involved in the neuroprotective effect of Rg1 on dopaminergic neurons against 6-OHDA toxicity.

There is accumulating evidence for an abundant expression of IGF-IR and ER in hippocampus. The interactions of IGF-1 and estradiol may promote neuroprotection under neurodegenerative conditions (Garcia-Segura et al., 2010). However, it is unclear if Rg1 exert neuroprotective effects in Aβ-induced cellular model for AD via IGF-IR and ER signaling pathways. Lesioning of sympathetic nerve pheochromocytoma cell line PC12 cells with Aβ is extensively used as an experimental model of AD (Wei et al., 2003). In the present study, we hypothesized that the IGF-IR-mediated pathway and ER-mediated pathway might be involved in the neuroprotective effect of Rg1 against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells.

2. Material and methods

2.1. Cell culture

PC12 cells (ATCC No. CRL-1721™) were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin 100 IU/mL, and streptomycin 100 μg/mL at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. When the cells reached 80–90% confluence, the medium was changed to 5% charcoal-stripped fetal bovine serum in phenol red-free DMEM for 24 h.

The cells were treated with Rg1 for 1 h after which it was replaced with media containing preaggregated $A\beta_{25-35}$ (purchased from Sigma, St. Louis, MO, USA) and Rg1. The $A\beta_{25-35}$ used in this study was dissolved in normal saline at a concentration of 1.0 mM and incubated in a 37 °C water bath for four days to induce aggregation. Ginsenoside Rg1 (purity > 98%) was obtained from the department of Organic Chemistry of Norman Bethune Medical University (Changchun, Jilin, China). For antagonist treatment, JB-1 (1 g/mL) (Bachem, Bubendorf, Switzerland) or ICI182,780 (0.1 M) (Tocris, Bristol, U.K.) was added to each group for 1 h before Rg1 treatment. All of Rg1, JB-1 and ICI182,780 were dissolved in ethanol.

2.2. Cell viability assay

The cells were treated with different dosages of preaggregated $A\beta_{25-35}$ (0.1, 1, 10, 20, 40 μM) for 72 h. For the protective effects assay, PC12 cells were treated with ginsenoside Rg1 (0.01, 1, 10 μM) for 1 h after which it was replaced with media containing 10 μM $A\beta_{25-35}$ and Rg1 for 72 h. For antagonist treatment, JB-1 (1 μg/mL) or ICI182,780 (0.1 μM) was added to each group for 1 h before Rg1 treatment. Cell viability was measured by the 3-[4, 5-dimethylthiazol 2-yl] 2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the medium was removed and replaced with 20 μL of tetrazolium (5 mg/mL, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS buffer). The plates were incubated for 4 h at 37 °C and followed by the addition of 100 μL dimethyl sulfoxide (DMSO). The multiwell plates were shaken for 15 s and the signals were detected by a microplate reader using a wavelength of 595 nm. Cell viability was expressed as a percentage of the control cells treated with vehicle and was designated as 100%.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) for Bcl-2 mRNA expression

PC12 cells were pretreated with ginsenoside Rg1 (0.01, 1, 10 μM) or vehicle for 1 h after which it was replaced with media containing $A\beta_{25-35}$ (10 μM) and Rg1. After 24 h treatment, total RNA was isolated from cells by using Trizol reagent according to the standard protocols. Total RNA (2 μg) was reverse-transcribed in 20 μL of a reaction mixture that contained reverse transcription buffer, deoxynucleotide triphosphate mixture, random primers and MultiScribe reverse transcriptase, using the high-capacity cDNA reverse transcription kit (Applied Biosys-tems Inc, Foster City, CA, USA), at 25 °C for 10 min, 37 °C or 2 h and 85 °C for 5 s. The sequences of the PCR primers for Bcl-2 and GAPDH were 5′-TGGGATGCCTTTGTGGAACTA-3′(Bcl-2 forward) and 5′-GCTGAT TTGACCATTTGCCTG-3′(Bcl-2 reverse), and 5′-TGGCATCGTGGAA GGGCTCAT-3′(GAPDH forward) and 5′-CCACCACCCTGTTGCTG TAAC-3′(GAPDH reverse). PCR was carried out in 20 μL reaction mixture containing 10 μL SYBR Green Super-mix (Bio-Rad Laboratories, Hercules, CA, USA) and 0.5 μL of cDNA template. The PCR was performed in an ABI 7900HT fast real-time PCR system (Applied Biosystems) using the following cycle parameters: 1 cycle of 95 °C for 1 min, and 40 cycles of 95 °C for 20 s, 56 °C (Bcl-2), or 56 °C (GAPDH) for 20 s and 72 °C for 18 s. Standard curves were generated using serially diluted solutions of cDNA derived from untreated cells. The target gene transcripts in each sample were normalized to the expression of a housekeeping gene GAPDH.

2.4. Flow cytometry

PC12 cells were treated with ginsenoside Rg1 (1 μM) in the presence or absence of JB-1 or ICI182,780 for 1 h after which it