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Research report

Neuroprotective effects of puerarin against beta-amyloid-induced neurotoxicity in PC12 cells via a PI3K-dependent signaling pathway

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ABSTRACT

Epidemiological data have indicated that estrogen replacement therapy (ERT) can decrease the risk of developing Alzheimer's disease (AD). Phytoestrogens have been proposed as potential alternatives to ERT. The aim of the present study was to assess the neuroprotective effects of puerarin, a phytoestrogen isolated from Pueraria lobata, against the toxicity of beta-amyloid (Aβ) in relation to the mitochondria-mediated cell death process, and to elucidate the role the activation of Akt and modulation of the pro- and antiapoptotic proteins in puerarin-induced neuroprotection. The present study shows that puerarin afforded protection against Aβ-induced toxicity through inhibiting apoptosis in PC12 cells. This result was also confirmed by the activated caspase-3 assay. P-Akt, Bcl-2 and p-Bad expression increased after pretreatment with puerarin in PC12 cells exposed to $A\beta_{25-35}$, whereas Bax expression and cytochrome *c* release decreased. Interestingly, these effects of puerarin against $A\beta_{25-35}$ insult were abolished by wortmannin, an inhibitor of PI3K phosphorylation. These findings suggest that puerarin prevent Aβ-induced neurotoxicity through inhibiting neuronal apoptosis, and might be a potential preventive or therapeutic agent for AD.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder afflicting an estimated 30 million people worldwide [2]. However, the cause of AD is still unknown and the treatment is therefore only palliative [27,38]. Postmenopausal women may have a greater risk of developing AD than men, perhaps due to lower endogenous estrogen levels following menopause [15]. AD is more prevalent in women than in men [25]. Loss of ovarian steroids, particularly estrogens, at the menopause may increase the susceptibility of the aging brain to neurodegenerative disorders and be a risk factor for development of AD [43]. There is also good clinical evidence that surgical removal of the ovaries before menopause increases the risk of dementia and cognitive impairment in women [40]. Epidemiological studies indicate that estrogen replacement therapy (ERT) lowers the risk of developing AD [13]. Estrogen therapy is one of the most compelling potential strategies for the prevention of AD. Strong biologic evidence supports the beneficial of estrogen on the brain, including neurotrophic effects, reducing beta-amyloid (Aβ) accumulation, enhancing neurotransmitter release and action, and protecting against oxidative damage [4,39,48]. In addition, both prospective and case-control studies found that women who took estrogen had up to a 50% lower risk of developing AD [18]. Furthermore, in vitro studies have demonstrated neuroprotective effects of estrogens against glutamate- and β-amyloid-induced neurotoxicity [31]. ERT in post-menopausal women has been linked to a higher incidence of uterine and breast cancer, especially after long-term use [30]. Consequently, the selective estrogen receptor modulators (SERMs) that exert tissue specific estrogenic effects may provide the benefits of ERT without the risks [3]. A group of natural SERMs are phytoestrogens, which are structurally similar to estrogen, and may serve as an alternative to ERT [32].

Although there are a few papers about the beneficial effects of phytoestrogens on memory or the central nervous system [20], the effects of phytoestrogens on the central nervous system in humans are poorly understood. Puerarin (for its structure, see Fig. 1), a naturally occurring isoflavone C-glycoside, was isolated from Pueraria lobota. Puerarin which has been classified as a phytoestrogen

Abbreviations: AD, Alzheimer's disease; ERT, estrogen replacement therapy; SERMs, selective estrogen receptor modulators; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraz-olium bromide; LDH, Lactate dehydrogenase; FACS, Fluorescence activated cell sorting; PBS, phosphate-buffered saline; PI, propidium iodide; Ct, threshold cycle; ELISA, Enzyme-linked immunosorbent assay; S.D., standard deviation; ANOVA, one way analysis of variance; p-Bad, Bcl-2 associated death agonist; PTP, permeability transition pore; JNK, c-jun N-terminal kinase.

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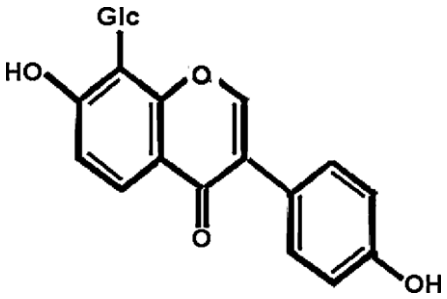

Fig. 1. Chemical structure of puerarin.

can be highly effective against angiocardiopathy and cerebrovascular diseases with properties of holding pharmacokinetics of rapid absorption from the intestine and presenting in brain organ tissue by specific transport pathways and low toxicity [34]. Zhang and co-workers have shown that puerarin could attenuate $A\beta_{25-35}$-induced PC12 cell injure and apoptosis and could also promote the survival of PC12 cells. Puerarin was also found to increase the Bcl-2/Bax ratio [47]. However, the upstream and downstream signaling components of regulation by puerarin of Bcl-2 family expression have also not been clearly resolved. It was recently reported that estrogen protects against Aβ-induced neurotoxicity via the activation of Akt [9]. Therefore, these findings led us to examine whether puerarin has a neuroprotective function, as estrogen does, and whether the effects are induced by suppressing neuronal apoptosis via the activation of PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Cell culture

PC12 cells, a rat pheochromocytoma, obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) were maintained in Dulbecco's modified Eagle medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 50 units/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 mg/ml streptomycin (Invitrogen). The cells were seeded in φ60 mm dishes (Nalge Nunc Int., Rochester, NY, USA) at 1×10^4 cells/cm^2 and maintained at 37 °C in a humidified atmosphere of 5% CO_2. For neuronal differentiation, the cells were cultured with appropriate concentration of 50 ng/ml rat NGF for 48 h [19]. Thereafter, the cells were washed repeatedly to remove NGF, and fresh medium replaced before all the experiments performed in the present study.

2.2. Determination of cell viability

Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described previously [14]. Briefly, the cells were cultured at a density 5×10^4 cells per well in growth medium for 24 h in 96-well plates, and then preincubated with or without 0.01–1000 μM or 100 μM puerarin (solubilized in 1,2-propanediol) (98% purity by HPLC; Sino-Herb Company, Xian, China), which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{25-35}$ (Sigma, St. Louis, MO, USA) prepared as described previously [33] for another 24 h or 0–48 h. Twenty five microliter/well of MTT solution (final concentration, 500 μg/ml) was added and cells were incubated at 37 °C for 4 h. Supernatants were then aspirated off and formazan crystals were dissolved with DMSO. The optical density of each well was determined at 570 nm using a microplate reader (Safire2, Tecan Group Ltd., Maennedorf, Switzerland).

2.3. Determination of cytotoxicity

PC12 cells were cultured as described above and then preincubated with or without puerarin at concentrations of 50 μM, 100 μM, and 200 μM, which was followed 1 h later by exposure to 20 μM aggregated $A\beta_{25-35}$ for 24 h. Lactate dehydrogenase (LDH) release was measured by cytotoxicity detection kit (Genmed, Westbury, NY) following the instructions provided by the manufacturer, and OD values were measured at 490 nm with a microplate reader (Safire2, Tecan Group Ltd., Maennedorf, Switzerland). Results were expressed as percentage of Triton X-100-induced LDH release.

2.4. Fluorescence activated cell sorting (FACS) analysis

The cells were cultured at a density 1.5×10^5 cells per well in growth medium for 24 h in 96-well plates, and then preincubated with 100 μM puerarin, which was followed 1 h later by exposure to 20 μM aggregated $A\beta_{25-35}$. Wortmannin (Calbiochem, San Diego, CA, USA), an inhibitor of PI3K phosphorylation, was added to cells 1 h prior to puerarin at a final concentration of 200 nM. Annexin V assays were done using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson, San Jose, CA). Cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in binding buffer before addition of Annexin V-FITC and propidium iodide (PI). Cells were vortexed and incubated for 15 min in the dark at room temperature before analysis using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, San Carlos, CA).

2.5. RNA isolation and real-time PCR

The cells were treated as described above for the FACS analysis, and harvested by scraping into ice cold PBS 24 h later. Total RNA was isolated from cells using RNAiso Reagent kit (Takara Biotechnology, Dalian, China), and cDNA was synthesized with ExScript™ RT kit (Takara Biotechnology, Dalian, China) according to the manufacturer' protocol. PCR were performed by using SYBR® Premix Ex Taq™ in an ABI7300 real-time PCR system (Applied Biosystems, CA). The following sequences were used as primers for real-time PCR amplification: Bax sense primer, 5′-AGA CAC CTG AGC TGA CCT TGG AG-3′, and Bax antisense primer, 5′-GTT GAA GTT GCC ATC AGC AAA CA; Bcl-2 sense primer, 5′-TGA ACC GGC ATC TGC ACA C-3′, and Bcl-2 antisense primer, 5′-CGT CTT CAG AGA CAG CCA GGA G-3′; and GAPDH sense primer, 5′-GAC AAC TTT GGC ATC GTG GA-3′and GAPDH antisense primer, 5′-ATG CAG GGA TGA TGT TCT GG-3′. The thermal profile was as follows: 1 cycle of 95 °C for 10 s; 40 cycles of 5 s at 95 °C and 31 s at 60 °C. Threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). The Ct represents the cycle number at which a fluorescent signal rises statistically above background. Real-time PCR assay was performed in triplicate for each sample to ensure reproducibility. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method [23]. The fold change in target gene cDNA relative to the GAPDH internal control was determined by:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}, \text{ where } \Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{GAPDH}) - (Ct_{\text{control}} - Ct_{GAPDH})$$

2.6. Western blot

The cells were treated as described above for the FACS analysis. Cytoplasm proteins were isolated from PC12 cells using Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China), and protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, China), then they were aliquoted and stored. One hundred microliter of supernatant was added to an equal volume of 2× SDS sample buffer and boiled for 5 min at 100 °C. The samples were then stored at −80 °C until analyzed. Equal amounts of protein (100 μg/lane) were separated by 15% SDS-polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose filter membrane. After blocking for 4 h in a solution of 8% nonfat dry milk in Tris-buffered saline containing 0.1% Tween (pH 7.6) at room temperature, membrane was then incubated overnight at 4 °C with primary antibody (caspase-3, phospho-Akt, and Akt antibody, Cell Signaling Technology Inc., Beverly, MA; other antibody, Santa Cruz Biotechnology, Santa Cruz, CA) in concentrations of 1:1000 (p-Akt), 1:1000 (Akt), 1:1500 (Bcl-2), 1:1500 (Bax), 1:1000 (p-Bad), 1:1000 (Bad), and 1:2500 (GAPDH) in Tris-buffered saline with 0.1% Tween 20 containing 8% nonfat dry milk. After washing four times, the membrane were incubated with Horseradish Peroxidase Labeled Anti-Mouse IgG (10000:1; Medical Biological Laboratory Co., Nagoya, Japan) at room temperature for 2 h and again washed four times. The blots were developed using an ECL western blotting kit (Amersham Biosciences, Piscataway, NJ, USA) as recommended by the manufacturer. GAPDH was probed as an internal control to confirm that an equal amount of protein was loaded in each lane. Band intensities were quantified by an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The cells were treated as described above for the FACS analysis. Cells were collected and fractionated. Cytosolic cytochrome *c* was determined by the Quantikine® rat/mouse cytochrome *c* assay kit (R&D systems, Minneapolis, MN, USA) within 96-well plates according to procedure given by the manufacturer. Cytosolic fractions were pipetted in triplicate onto a microplate precoated with rat/mouse cytochrome *c*. After a 2 h incubation and washing, substrate solution was added to each well. The reaction was stopped after 30 min, and the optical density was measured at 450 nm, corrected at 540 nm, using a microplate reader (Safire2, Tecan Group Ltd., Maennedorf, Switzerland). Cytochrome *c* concentrations expressed as nanograns/milliliter were extrapolated from the standard curves generated using reconstitute the r/m cytochrome *c* standard.

2.8. Statistical analysis

All values in the figures of present study indicate means ± standard deviation (S.D.), and all determinations were repeated three times. The one way analysis