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Attenuation of $A\beta_{25-35}$-induced parallel autophagic and apoptotic cell death by gypenoside XVII through the estrogen receptor-dependent activation of Nrf2/ARE pathways

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ABSTRACT

Amyloid-beta (Aβ) has a pivotal function in the pathogenesis of Alzheimer's disease. To investigate Aβ neurotoxicity, we used an in vitro model that involves $A\beta_{25-35}$-induced cell death in the nerve growth factor-induced differentiation of PC12 cells. $A\beta_{25-35}$ (20 μM) treatment for 24 h caused apoptotic cell death, as evidenced by significant cell viability reduction, LDH release, phosphatidylserine externalization, mitochondrial membrane potential disruption, cytochrome c release, caspase-3 activation, PARP cleavage, and DNA fragmentation in PC12 cells. $A\beta_{25-35}$ treatment led to autophagic cell death, as evidenced by augmented GFP-LC3 puncta, conversion of LC3-I to LC3-II, and increased LC3-II/LC3-I ratio. $A\beta_{25-35}$ treatment induced oxidative stress, as evidenced by intracellular ROS accumulation and increased production of mitochondrial superoxide, malondialdehyde, protein carbonyl, and 8-OHdG. Phytoestrogens have been proved to be protective against Aβ-induced neurotoxicity and regarded as relatively safe targets for AD drug development. Gypenoside XVII (GP-17) is a novel phytoestrogen isolated from *Gynostemma pentaphyllum* or *Panax notoginseng*. Pretreatment with GP-17 (10 μM) for 12 h increased estrogen response element reporter activity, activated PI3K/Akt pathways, inhibited GSK-3β, induced Nrf2 nuclear translocation, augmented antioxidant responsive element enhancer activity, upregulated heme oxygenase 1 (HO-1) expression and activity, and provided protective effects against $A\beta_{25-35}$-induced neurotoxicity, including oxidative stress, apoptosis, and autophagic cell death. In conclusion, GP-17 conferred protection against $A\beta_{25-35}$-induced neurotoxicity through estrogen receptor-dependent activation of PI3K/Akt pathways, inactivation of GSK-3β and activation of Nrf2/ARE/HO-1 pathways. This finding might provide novel insights into understanding the mechanism for neuroprotective effects of phytoestrogens or gypenosides.

Introduction

Alzheimer's disease (AD) is the most common form of senile dementia. Currently, more than 35 million patients are estimated to be affected worldwide (Querfurth and LaFerla, 2010). The pathological hallmarks of AD are senile plaques, neurofibrillary tangles, and progressive neuron loss (Serrano-Pozo et al., 2011). Although the etiology of AD is not fully understood, numerous experimental and clinical observations show that amyloid-β peptide (Aβ), which is the major component of senile plaques, elicits neurotoxicity and has a pivotal function in AD pathogenesis (Deshpande et al., 2006; Schaeffer et al., 2011). Inhibition of Aβ-induced neurotoxicity might provide neuroprotective effects against AD. However, the molecular mechanisms underlying Aβ-induced neurotoxicity are largely unknown.

Several mechanisms including oxidative stress, apoptosis, and autophagy have been implicated in Aβ-induced neurotoxicity (G. Chen et al., 2013; Liu and Zhao, 2004; H. Wang et al., 2010; Yan et al., 2013). Oxidative stress reflects a cellular redox imbalance in which production of reactive oxygen species (ROS) overwhelms the endogenous antioxidant enzyme system. As a consequence of oxidative stress, lipid peroxidation, protein and DNA oxidation, and alterations in redox state contribute to Aβ-induced neuronal death. Apoptosis and autophagy have been involved in Aβ-induced neuronal death (Huang et al., 2012; H. Wang et al., 2010). Apoptosis is the process of programmed cell death, which is generally characterized by cell shrinkage, chromatin

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condensation, and DNA fragmentation, in which mitochondrial dysfunction and caspase activation have key functions. Autophagy is a self-degradation process that involves cell degradation of misfolded or aggregated proteins and damaged organelles. This process is accompanied by progressive development of vesicle structures from autophagosomes to autolysosomes (Klionsky et al., 2012). Although autophagy is generally considered as a survival mechanism, deregulation of autophagy has been implicated in Aβ-induced neuronal death.

Current therapies for AD such as acetylcholinesterase inhibitors rely on only alleviating symptoms (Roberts et al., 2012). When timely initiated at the onset of menopause, estrogen therapy can potentially counteract the cognitive changes and reduce the AD risk. Various experimental and epidemiological studies have supported neuroprotection of estrogen against AD (Brann et al., 2007; Lee et al., 2013; Napolitano et al., 2013). However, the application of estrogen therapy has been hampered by its potential adverse outcomes, such as breast and uterine cancer (Grady et al., 1995). Therefore, it is important to explore a novel pharmacological therapy that can mechanistically delay or prevent the onset and progress of AD but circumvent the potential risks associated with estrogen therapy. In search of alternatives to estrogen therapy, estrogen analog phytoestrogens are regarded as relatively safe targets for AD drug development.

Phytoestrogens are non-steroidal plant compounds that can bind weakly to estrogen receptors and exert estrogenic properties (Cos et al., 2003). Experimental, clinical, or epidemiological studies suggest that phytoestrogens have protective effects against Aβ-induced neurotoxicity and AD (W.F. Chen et al., 2013; Sumien et al., 2013; Zhao et al., 2013). The mechanisms for neuroprotection of phytoestrogens might be associated with estrogen receptor-dependent genomic and non-genomic actions. The classical mechanism of genomic action involves estrogen receptors that bind to estrogen response elements (EREs) located in the promoters of target genes. Estrogen receptor-dependent non-genomic actions occur by interacting with other transcription factors or by activating intracellular signaling pathways (Bjornstrom and Sjoberg, 2005), such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and nuclear factor-erythroid 2-related factor 2/antioxidant responsive element (Nrf2/ARE) pathways. Both PI3K/Akt and Nrf2/ARE pathways have been implicated in oxidative stress, apoptosis, and autophagy (Tufekci et al., 2011; Wang et al., 2008). This finding has raised the possibility that the regulation of these two pathways by phytoestrogens may help in attenuating Aβ-induced neurotoxicity.

GP-17, the structure of which (shown in Fig. 1) is similar to that of estradiol (E2), is a novel phytoestrogen found in gypenosides. Gypenosides are the main saponins isolated from *Gynostemma pentaphyllum*, which have been used for various medicinal purposes in traditional Chinese medicines for many years. Gypenosides are reported to improve cognitive impairment induced by chronic cerebral hypoperfusion in rats by suppressing oxidative stress (Zhang et al., 2011). Gypenosides are also reported to protect the brain against cerebral ischemia and confer neuroprotection against oxidative injury induced by MPTP or glutamate (P. Wang et al., 2010). However, whether GP-17 has neuroprotective effect is poorly understood.

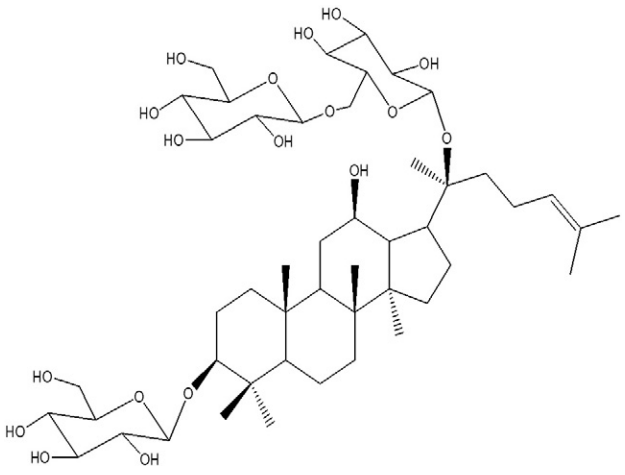

Fig. 1. The chemical structure of GP-17.

In the present study, an extensively used in vitro model that involves $A\beta_{25-35}$-induced neurotoxicity in NGF-induced differentiation of PC12 cells was employed to evaluate the potential neuroprotective effect of GP-17. For the first time, the significant neuroprotective effects of GP-17 against oxidative stress, autophagy, and apoptosis induced by $A\beta_{25-35}$ are elucidated.

Methods

Materials. GP-17 (molecular weight = 947.158; purity > 98%) was purchased from Shanghai Winherb Medical S&T Development (China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum, Opti-MEM, and phosphate-buffered saline (PBS) were purchased from Gibco (NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), total ROS detection kits, and HO-1 ELISA kits were purchased from Enzo Life Sciences (PA, USA). The protein carbonyl ELISA kits, 8-OHdG ELISA kits, and pCMV-GFP-LC3 expression vector were obtained from Cell Biolabs (CA, USA). Cell nuclear protein extraction kits, primary and secondary antibodies, Nrf2 small interfering RNA (siRNA), control siRNA, and tin protoporphyrin IX dichloride (Snpp) were purchased from Santa Cruz Biotechnology (CA, USA). The bicinchoninic acid assay kits and the enhanced chemiluminescence Western blot detection kits were purchased from Pierce Biotechnology (IL, USA). Dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI), lithium chloride (LiCl), estradiol, D,L-sulforaphane, N-acetyl-L-cysteine (NAC), and ICI-182,780 were purchased from Sigma–Aldrich (MO, USA).

Cell culture and drug preparation. PC12 cells, which belong to a rat pheochromocytoma cell line, were obtained from the Cell Resource Center of the Institute of Basic Medical Sciences, Peking Union Medical College and Chinese Academy of Medical Sciences (Beijing, China). PC12 cells were cultured in DMEM supplemented with 10% horse serum and 5% FBS at 37 °C in 5% CO_2 and 95% atmosphere. In all experiments, PC12 cells were differentiated into neural cells by incubating with nerve growth factor (NGF, 50 ng/mL; New England Biolabs, MA, USA) containing medium for 7 d prior to being subjected to various treatments. NGF-induced differentiation of PC12 cells was determined by immunofluorescence studies using antibodies against the neuron-specific marker microtubule-associated protein 2 (MAP2). GP-17 stock solution (1 M) was prepared in DMSO and diluted with fresh complete medium immediately before use. The control cells were treated with DMSO (final concentration was <0.1%). $A\beta_{25-35}$ (1 mM) was dissolved in deionized distilled water and diluted to desired concentrations immediately before use. In specified experiments, PC12 cells were pretreated with ICI-182,780 (1 μM), LY294,002 (20 μM; Calbiochem, CA, USA), or LiCl (20 mM) for 1 h followed by incubation with GP-17 or $A\beta_{25-35}$.

Analysis of cell viability. Cell viability of PC12 cells was determined by MTT reduction assay. PC12 cells (5×10^4 cells/well) were plated in poly-L-lysine-coated 96-well plates and allowed to attach overnight. The cells were incubated with MTT solution (1 mg/mL final concentration) at 37 °C for 4 h at the end of different treatments. To dissolve formazan crystals, the MTT solution was replaced with 100 μL of DMSO. After 10 min shaking, the absorbance was detected at 570 nm on a microplate reader (SpectraFluor, Tecan, Sunrise, Austria). Cell viability was expressed as the percentage of the control.