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The neuroprotective effects of phytoestrogen α-zearalanol on β-amyloid-induced toxicity in differentiated PC-12 cells

Yilong Dong [a,b], Nan Yang [a], Yanyong Liu [a], Qing Li [a], Pingping Zuo [a,*]

[a] Institute of Basic Science, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100005, China
[b] School of Life Science, Yunnan University, 2 Cuihu Bei Road, Kunming, Yunnan 650091, China

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ABSTRACT

Although favorable effects of estrogen replacement therapy on Alzheimer's disease on postmenopausal women have been recognized, an associated increased incidence of uterine and breast tumors has jeopardized the clinical use of estrogen. Phytoestrogen α-zearalanol (α-ZAL) is a reductive product of the Gibberella zeae metabolite and abundant in plants and vegetables, which has been shown to protect cell injury with low side-effects on uterine and breast. This study was designed to evaluate the neuroprotective effects of α-ZAL, on the cultured differentiated PC-12 cells, while 17β-estradiol (17β-E2) has been used as an estrogen positive control. Following a 24 h exposure of the cells to amyloid β-peptide fragment 25–35 ($A\beta_{25-35}$), a significant reduction in cell survival and activities of total superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as increased of malondialdehyde (MDA) were observed. However, preincubation of the cells with α-ZAL or 17β-E2 prior to $A\beta_{25-35}$ exposure elevated the cell survival and SOD and GSH-Px activities, and decreased the level of MDA. In addition, $A\beta_{25-35}$ caused a significant cell apoptosis and increased apoptotic rate, accompanied by decreasing of bcl-2 expression and increasing bax, caspase-3 expression, pretreatment of the cells with α-ZAL or 17β-E2 ameliorated these changes induced by $A\beta_{25-35}$. Taken together, these data indicated that the phytoestrogen α-ZAL may effectively antagonize $A\beta_{25-35}$-induced cell toxicity by attenuating oxidative stress and apoptotic cell death, in a manner similar to 17β-E2. Our results suggested that α-ZAL can be used as a potential substitute of 17β-E2 in postmenopausal women for Alzheimer's disease prevention.

1. Introduction

Alzheimer's disease is a neurodegenerative disorder characterized clinically by progressive dementia and pathologically by intraneuronal neurofibrillary tangles, extracellular deposition of amyloid β peptides (Aβ) and phosphorylation of tau protein (Forman et al., 2004; Mattson, 2004). Although the cause of Alzheimer's disease is still unknown, researches have indicated that postmenopausal women appear to be at an increased risk for Alzheimer's disease compared with the same age men or premenopausal women (Miech et al., 2002; Zandi et al., 2002). Postmenopausal depletion of endogenous estrogens may contribute to this risk. Several studies have reported that estrogen replacement therapy may reduce the risk of developing Alzheimer's disease in postmenopausal women (Asthana et al., 1999; Canderelli et al., 2007; Ohkura et al., 1994; Shaywitz et al., 2003; Valen-Sendstad et al., 2010). Estrogens may exert several neuroprotective effects on the brain, including inhibition of Aβ formation, diminishing hyperphosphorylation of tau protein, reduction of oxidative stress-related cell damage, and protection of neurons against the noxious consequences of chronic inflammatory reaction (Simpkins et al., 2009). However, the fact that estrogen may predispose women to a much higher incidence of breast and endometrial cancers has undoubtedly compromised or jeopardized the clinical application of estrogen (Chlebowski et al., 2010). Thus, the search for safe and effective estrogen substitutes becomes a practical issue.

Recently, a plant-derived phytoestrogen, α-zearalanol (α-ZAL) has been proposed as a potential replacement for estrogen. α-ZAL is a reductive product of the Gibberella zeae metabolite zearalenone (ZEN) (Fig. 1), which is abundant in plants and vegetables including soybean, wheat, grape, radish, celery, spinach and apple (Zöllner et al., 2000). α-ZAL is rapidly metabolized in the body with few residues left in organs such as muscle, heart, liver, pancreas, kidney, and blood (Kleinova et al., 2002). It has been observed to protect human umbilical vein endothelial cell against damage induced by homocysteine or oxidized low density lipoprotein (oxLDL) via its antioxidant property (Duan et al., 2008; Xu et al., 2008). In addition, Zheng and colleagues have reported that α-ZAL may inhibit proton F0F1-ATPase activity of rat brain mitochondrial, which leads to protective actions and antitumor property. (Zheng and Ramirez, 1999) Also, there were evidences shown that the effect of α-ZAL on breast oncogene expression (Deng et al., 2010) and uterine

* Corresponding author at: Department of Pharmacology, Institute of Basic Science, Chinese Academy of Medical Sciences, 5 Dong Dan San Tiao, Beijing 100005, China. Tel.: +86 10 6529 6404; fax: +86 10 6529 6404.
E-mail address: zuopp_pumc@126.com (P. Zuo).

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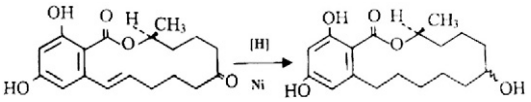

Fig. 1. Chemical structure of zearalenone (ZEN, left panel) and α-zearalanol (α-ZAL, right panel).

overgrowth is less than estrogen (Dai et al., 2004a); furthermore, α-ZAL may increase the gene expression of anti-tumor BIN1 in breast tissue (Deng et al., 2004), meaning that α-ZAL was able to alleviate the incidence of mammary gland tumor. Previously, our published results have shown that α-ZAL may effectively alleviate synapse loss and neuronal damage induced by Aβ just like estrogen (Dong et al., 2006, 2007), indicating that α-ZAL can be used as a "safe-estrogen" in postmenopausal women for Alzheimer's disease prevention dependent on its low side-effect compared with estrogen. However, the mechanism of α-ZAL against Aβ-induced cytoxicity is still unclear. Therefore, in the present study, we investigated the effect of α-ZAL on $A\beta_{25-35}$-induced neurotoxicity in differentiated PC-12 cells in vitro and underlying molecular mechanisms when 17β-estradiol (17β-E2) has been used as an estrogen positive control.

2. Materials and methods

2.1. Materials

$A\beta_{25-35}$, 17β-E2, nerve growth factor (NGF), MTT, RNase A and propidium iodide (PI) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum (HS) were obtained from Gibco Invitrogen Corporation (Carlsbad, CA, USA). The protease inhibitor mixture and BCA Protein Assay Kit was purchased from Pierce Biotechnology (Rockford, IL, USA). The reagent kits for the measurement of MDA, SOD, and GSH-Px were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Bcl-2, bax, caspase-3 and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). α-ZAL was obtained as a gift from Prof. Shunling Dai at Perking Union Medical College. All other chemicals used were of the highest grade commercially available.

2.2. Cell culture and treatment

Rat pheochromocytoma cells (PC12 cells, obtained from Cell Center, Institute of Basic Science, Chinese Academy of Medical Sciences) were cultured in DMEM supplemented with 5% FBS, 10% HS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37 °C and grown until 80% confluence. Cells were differentiated with 50 ng/ml NGF for 5 days and used for a maximum of 25 passages. α-ZAL or 17β-E2 was dissolved in ethanol and added to cell culture media at designated concentrations, the final concentration of ethanol ≤ 0.1%. 12 h later, cells were exposed to $A\beta_{25-35}$ for 24 h. Then, the cells will be collected for further research. Prior to α-ZAL or 17β-E2 treatment, the media were replaced with fresh DMEM without FBS in order to remove endogenous estrogen. $A\beta_{25-35}$ peptide stock solution of 1 mM was prepared in sterile distilled water, stored at −20 °C, and incubated for 72 h at 37 °C to aggregate before use.

2.3. Cell viability assay

Cells were plated in 96-well plates, after various treatments, cell viability was determined by MTT assay. MTT was applied to the cultures at a final concentration of 0.5 mg/ml for 4 h at 37 °C. The medium was then aspirated, and 100 μl dimethyl sulfoxide (DMSO) was added to solubilize the colored formazan product. Absorbance was determined at 578 nm on a scanning multiwell plate reader (Dynatech Lab, USA) after agitating the plates.

2.4. Measurement of MDA content and antioxidant enzyme activities

For assay of lipid peroxide and antioxidants, the cultures were washed with ice-cold PBS and then pooled in 0.1 M PBS–0.05 mM EDTA-buffered solution and homogenized. The homogenate was centrifuged at 11,000 × *g* for 20 min at 4 °C, after which the protein concentration was determined by the BCA Protein Assay Kit, using bovine serum albumin (BSA) as a reference standard. The supernates were collected and stored at −80 °C until use. The levels of MDA and antioxidant enzyme activities include total SOD and GSH-Px was determined according to the instructions for the reagent kits.

2.5. Transmission electron microscopy

For electron microscopic analysis of apoptosis, the cells were harvested by trypsinization and washed twice with cold PBS, then fixed in 2.5% glutaraldehyde for 2 h at room temperature. After being washed with PBS, the cells were post-fixed in 1% osmium tetroxide containing 0.1% potassium ferricyanide for 1 h at 4 °C, dehydrated through graded alcohol, and embedded in Epon 812 for subsequent sectioning. The ultra-thin sections (65 nm) were stained with uranyl acetate and lead citrate, and examined under transmission electron microscope.

2.6. Flow cytometry assay

For flow cytometry quantitative analysis of apoptosis, the cells were harvested by trypsinization and washed twice with cold PBS, then fixed in 70% ethanol for 30 min at 4 °C. After being washed with PBS, the cells were collected by centrifugation at 800 × *g* for 5 min at 4 °C, then resuspended in a solution containing 10 g/l RNase A and 20 mg/l PI at room temperature for 30 min. Specimens were analyzed using flow cytometry. The cells in the subdiploid peak were considered apoptotic.

2.7. Western blotting

Cells after treatment were washed three times with cold PBS and lysed using cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM ethylenediamine tetraacetic acid, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor mixture, then centrifuged at 11,000 × *g* for 10 min at 4 °C. The supernatant will be collected and protein concentration was estimated by BCA Protein Assay Kit, using BSA as the standard. Equal amounts of protein were subjected to SDS-PAGE. The proteins were then transferred to nitrocellulose membranes. After transfer, the membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% non-fat-milk for 2 h at room temperature and then overnight at 4 °C with primary antibodies: anti-bcl-2 (1:1000), anti-bax (1:500), anti-caspase-3 (1:500) and anti-GAPDH (1:2000). After washing, the membranes were incubated with goatradish peroxidase-conjugated secondary antibody (1:5000), followed by detection using enhanced chemiluminescence. Scanned images of the developed blots were quantified using densitometry functions in Image-Pro Express 4.0.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). Statistical analyses were performed with one-way ANOVA followed by SPSS software. A criterion for statistical confidence of $P \leq 0.05$ (two tailed) was adopted. Data from triplicate performances and all experiments were performed using 3 separate cultures to confirm reproducibility.