

Protective effects of phytoestrogen α -zearalanol on beta amyloid_{25–35} induced oxidative damage in cultured rat hippocampal neurons

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Abstract Although experimental evidence has shown that the neuroprotective effect from estrogen may benefit postmenopausal women, but the clinical use of estrogen was limited by the risk of increasing the cases of mammary and endometrial cancer. This study was designed to evaluate the neuroprotective effects of a novel phytoestrogen α -zearalanol (α -ZAL), on the cultured rat hippocampal neurons. 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. Preincubation of the cells with α -ZAL or 17 β -estradiol (17 β -E2) prior to $A\beta_{25-35}$ exposure elevated the cell survival and SOD and GSH-Px activities, and decreased the level of MDA. These data suggested that the phytoestrogen α -ZAL, like estrogen, may effectively antagonize $A\beta_{25-35}$ -induced cell toxicity, which might be beneficial for neurons.

Keywords α -ZAL · Amyloid β -peptide · Superoxide dismutase · Glutathione peroxidase · Malondialdehyde · Hippocampal neurons

Introduction

Excessive accumulation of amyloid β -peptide ($A\beta$) in the brain is a major characteristic of Alzheimer's disease (AD) and a possible cause of neurodegeneration [1]. Although the mechanism by which $A\beta$ induces neuronal cell death remains controversial and unclear, but there is evidence suggesting that oxidative damage plays a key role [2, 3]. Due to neurotoxic effects of $A\beta$ are at least in part mediated by free radicals and some antioxidants have been proved to rescue cells from $A\beta$ toxicity [4], lessen the oxidative stress induced by $A\beta$ may be beneficial to delay process of AD.

Estrogens have an important role in maintaining neural functions and in protection against damage in the normal adult brain. Estrogens facilitate axonal sprouting and neuronal repair, reduce neuronal injury and enhance synaptic transmission and neurogenesis [5, 6]. Moreover, in vitro studies have shown that neuronal cell death induced by $A\beta$ is protected by estrogen [7, 8]. These beneficial effects have led to use estrogen replacement therapy as an effective method to reduce the risk and severity of AD in postmenopausal women who burden a higher incidence of AD than men of the same age or premenopausal women [9, 10]. However estrogen therapy has been found to increase the risk of breast and endometrial cancer [11]. The adverse effect has undoubtedly jeopardized the clinical application of estrogen. Therefore, the search for safe preventive and therapeutic strategies is in high demand to reconcile the estrogen deficiency-related neurodegenerative diseases in postmenopausal women.

Recently a plant-derived phytoestrogen, α -zearalanol (α -ZAL) have shown some promise as a replacement for estrogen to limit the risk of carcinoma. α -ZAL is a reductive product of the *Gibberella zeae* metabolite, which

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is abundant in plants and vegetables including soybean, wheat, rape, radish, celery, spinach, and apple [12]. α -ZAL is rapidly metabolized in the body with few residues left in organs such as muscle, heart, liver, pancreas, kidney, and blood [13]. It has been reported that α -ZAL is safe and efficient, used as food additives in animal husbandry [14]. More important, α -ZAL was able to alleviate DMBA-induced mammary gland tumor formation, and decrease the incidence of estrogen-dependent cancer [15]. Mounting evidence has shown that α -ZAL possesses similar physiological properties of estrogen such as the prevention of atherosclerosis, osteoporosis, but less side-effect especially on the uterine and mammary glands [16]. Our published results show that α -ZAL may effectively abate neurons loss in hippocampus which was induced by estrogen deficiency, analogous to that of estrogen [17]. Nevertheless, there is rare research in the relationship between α -ZAL and $A\beta$. The present study was designed to evaluate the neuroprotective of α -ZAL against $A\beta$ induced cells damage in cultured rat hippocampal neurons when used 17β -E2 as positive control.

Materials and methods

Cell culture

Hippocampi from Sprague–Dawley rats at embryonic day 18 were dissected, and primary hippocampal cultures were prepared as described previously with minor alterations [18]. Briefly, hippocampal tissue was mechanically dissociated after incubation with trypsin (0.25%) and DNAase (50 μ g/ml), then the cells were plated in a polylysine-coated tissue culture. The cells were grown in a humidified incubator with 5%CO₂ 95%air at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum for 2 h, after which the cultures were changed to neurobasal medium supplemented with B27 (Invitrogen-Gibco BRL, Carlsbad, CA, USA), 100 μ g/ml streptomycin, and 100 U/ml penicillin. Cells were treated for 24 h with 2 μ M 1- β -Darabinofuranosylcytosineon day 3 to reduce the number of proliferating non-neuronal cells. Animal handling was done in accordance with the guidelines published by the Laboratory Animal Center of the Chinese Academy of Medical Sciences.

Preparation of the $A\beta$ fibrils

The amyloid β -peptide fragment 25–35 ($A\beta_{25-35}$) peptide (Bachem, Torrance, CA, USA) was subjected to stirring aggregation as described previously [19]. In brief, aliquots of peptide stock (70 nmol in 20 μ l of dimethyl sulfoxide)

were added to PBS (10 mM phosphate pH 7.4, containing 137 mM NaCl and 27 mM KCl; 725 μ l total volume), and the solutions were stirred continuously at room temperature for 48 h. Then, preformed fibrils were washed three times with PBS, concentrated by centrifugation (20,000 g for 30 min), and resuspended in DMEM at 1 mM.

Experimental treatments

For the assessment of cell viability, cells were plated into 96-well culture plates at 10⁴ cells per well. For the assessment of malondialdehyde (MDA), total superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), cells were plated into 6-well culture plates at approximately 3 \times 10⁵ cells per well. After maintaining the cultured neurons for 7d, α -ZAL or 17β -E2 was added to the cultures by bath application. α -ZAL (gift from Prof. Shunling Dai at Perking Union Medical College) and 17β -E2 (Sigma, St. Louis, MO, USA) were dissolved in dehydrated alcohol. Control cultures received an equal amount of alcohol. About 12 h after α -ZAL or 17β -E2 incubation, $A\beta_{25-35}$ at the final concentration of 10 μ M was bath-applied to the cultured cells. About 24 h after $A\beta_{25-35}$ application, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dimethyl-tetrazolium bromide (MTT, Sigma) reduction was used to evaluate cell survival. MDA, SOD, and GSH-Px were determined to evaluate the effects of α -ZAL on antioxidant system.

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 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.

Determination of MDA, total SOD, and GSH-Px

The cultures were washed with ice-cold PBS and dissociated by 0.125% trypsin, 10⁶ cells were lysed in 0.1 M PBS-5 μ M EDTA-0.1% SDS-buffered solution and homogenized. The homogenate was centrifuged at 13,000 g for 20 min (4°C). The supernate were collected immediately and stored at –80°C until use. The levels of MDA, total SOD, and GSH-Px were determined by a commercially available kit (NanJingJianCheng Bio Inst, Nanjing, China). All experiments were performed using three separate cultures to confirm reproducibility.

MDA assay

Lipid peroxidation products MDA was determined by a photometrical method that is based on the method of Esterbauer and Cheeseman [20]. Chromogenic reagent reacts with MDA and yields a stable chromophore with maximal absorbance at 532 nm. Cell homogenates were added to a chromogenic reagent and incubated at 95°C for 60 min. After the samples were cooled off, absorbance was read at 532 nm.

SOD activity

SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O_2^- generated by the xanthine/xanthine oxidase system [21]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate.

GSH-Px activity

The assay of GSH-Px is based on the reaction described by Paglia and Valentine [22]. Its activity was determined by quantifying the rate of oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) by the hydrogen peroxide. A yellow product with absorbance at 412 nm could be formed as GSH reacts with 5,5'-dithiobis (2-nitrobenzoic acid). An enzyme unit of activity was defined as a decrease in 1 mM GSH per minute after the decrease in 1 mM GSH per minute of non-enzymatic reaction was subtracted.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical analyses were performed with one-way ANOVA followed by SPSS software (SPSS Science, Chicago, IL, USA). A criterion for statistical confidence of $P \leq 0.05$ (two tailed) was adopted.

Results

Morphological observation

As shown in Fig. 1, following 24-h exposure of the cells to 10 μ M $A\beta_{25-35}$, most cells lost neurites and demonstrated cell body atrophy and some of which were lysed or replaced by debris. In contrast, culture exposure to the same concentration of $A\beta_{25-35}$ in the presence of α -ZAL or

17 β -E2 (10^{-7} M, respectively) appear remarkably preserved, indicating α -ZAL and 17 β -E2 has a significant cytoprotective effects against $A\beta_{25-35}$ insult.

Effect of α -ZAL on $A\beta$ -induced cell death

Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly to the living cell number [23]. We examined the dose-dependent effect of α -ZAL on $A\beta_{25-35}$ -induced cell death. Cell survival as determined by MTT reduction was markedly decreased after hippocampal neurons were exposed to $A\beta_{25-35}$. However, when the cells were pre-incubated with α -ZAL at 10^{-8} M or more significantly reduced cell death induced by $A\beta_{25-35}$, and the effects plateaued at 10^{-7} M. The protection effect by α -ZAL was not been found at the concentration of 10^{-9} M, although 17 β -E2 might be against the damage at 10^{-9} M which was induced by $A\beta_{25-35}$, but the protection from α -ZAL or 17 β -E2 has no statistical difference at the range of 10^{-8} – 10^{-6} M. As shown in Fig. 2, the marked $A\beta_{25-35}$ -induced cell death occurred in these cultured neurons and α -ZAL protected them.

Evaluation of oxidative stress and antioxidants

The results of the observed MDA content and total SOD, GSH-Px specific activity in different groups including hormone treated are presented in Fig. 3. Treatment with $A\beta_{25-35}$ enhanced MDA contents in cultured neurons, these changes were lessened by treating with α -ZAL and 17 β -E2. $A\beta_{25-35}$ caused significant decreased SOD and GSH-Px specific activity, α -ZAL and 17 β -E2 treatment dose-dependently attenuated $A\beta$ -induced decreases in SOD and GSH-Px specific activity. Although compared with control group, this altered MDA content and SOD, GSH-Px activity was significant when pretreatment cultured neurons with 17 β -E2 at the level of 10^{-9} M, but there was no evident change when pretreatment neurons with equivalent doses of α -ZAL.

Discussion

The results of present study demonstrated that $A\beta_{25-35}$ caused significant toxicity in cultured hippocampal neurons, and α -ZAL protected cells against $A\beta_{25-35}$ -induced injury. α -ZAL prevents the decrease in SOD and GSH-Px and inhibited overproduction of MDA caused by $A\beta_{25-35}$, in a manner similar to that of 17 β -E2.

There is a confluence of opinion that cellular events involving oxidative stress may be one basic pathway leading to cell degeneration. Several independent lines of

Fig. 1 Effects of α -ZAL and 17β -E2 on hippocampal neurons injury induced by $A\beta_{25-35}$. (a) Control cells. (b) Cells exposed to $10\ \mu\text{M}$ $A\beta_{25-35}$ for 24 h. Part of the cells loss neuritis even lysed. (c) and (d) Cells were pre-incubated with 10^{-7} M α -ZAL or 17β -E2 for 12 h, and exposed to $10\ \mu\text{M}$ $A\beta_{25-35}$ for 24 h. ($\times 400$)

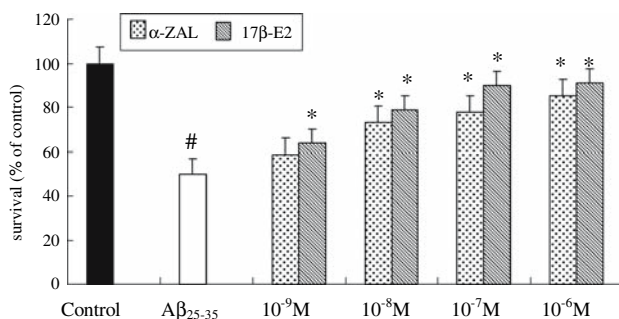
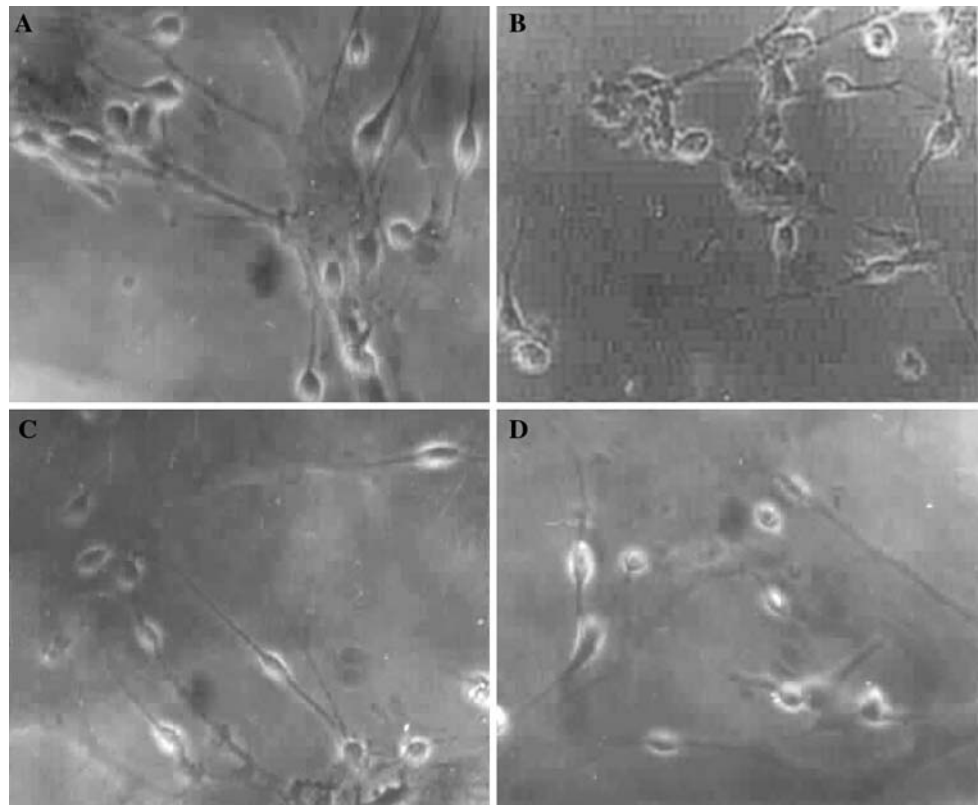


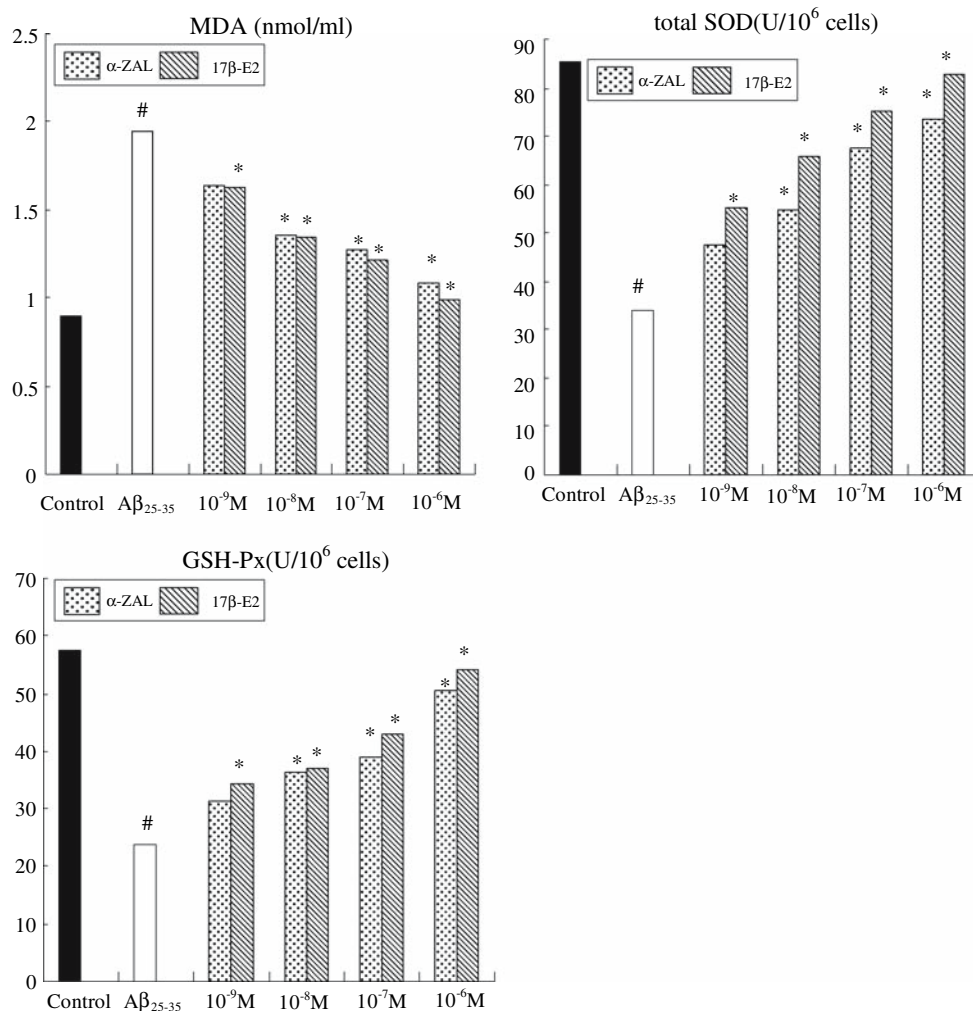
Fig. 2 Effects of α -ZAL and 17β -E2 on the toxicity induced by $A\beta_{25-35}$ in hippocampal neurons. Cell survival was assessed by measuring the MTT reduction. The data were expressed as percent of control value. # $P < 0.05$ vs. Control, * $P < 0.05$ vs. $A\beta_{25-35}$ group ($n = 6$)

investigation have now converged to suggest that increased oxidative stress and disturbed defensive mechanisms occur in the brain of AD victim, which might result in a self-propagating cascade of neurodegenerative events [2, 3]. To assess oxidative stress induced by $A\beta_{25-35}$, we choose to monitor MDA, an end product of lipid peroxidation, MDA alters the structure and function of the cellular membrane and block cellular metabolism leading to cytotoxicity [24], it was also considered as a late biomarker of oxidative stress and cellular damage [25]. Lipid degradation and consequently our present study showed that $A\beta_{25-35}$ caused a marked decrease in cell survival, which was accompanied

by increased oxidative stress as shown by accumulation of MDA. These results of cellular cytotoxicity and MDA level by $A\beta_{25-35}$ treatments are in good agreement with that of Jeong et al. [26]. However, increase in cell death and MDA level induced by $A\beta_{25-35}$ was significantly attenuated when the cells were pretreatment with α -ZAL (10^{-8} – 10^{-6} M) and 17β -E2 (10^{-9} – 10^{-6} M). These results suggest that α -ZAL protects hippocampal neurons against oxidative stress-induced toxicity.

Cells are often equipped with several antioxidants, such as SOD and GSH-Px, along with other non-enzymatic antioxidants, include α -tocopherol, ascorbate, and cysteine, serve as detoxifying system to prevent damage caused by reactive oxygen species which formed as byproducts of respiration and oxidative metabolism, and, among these, SOD and GSH-Px play a pivotal role. SOD act as a first cell defense against oxidative stress, catalysing the dismutation of O_2^- to H_2O_2 . High level of SOD activity protects neurons and other neuronal cell types against oxidative stress and nerve growth factor deprivation [27]. The down regulation of SOD has been shown to accelerate spontaneous cell death [28]. Another study reported that the over production of SOD prevents the brain mitochondrial respiratory dysfunction induced by glutathione depletion [29]. GSH-Px is the most prevalent low-molecular weight antioxidant within cells. GSH-Px protects cellular constituents from oxidative damage by reacting directly with oxidants or as

Fig. 3 Effects of α -ZAL and 17β -E2 on MDA content and total SOD, GSH-Px specific activity induced by $A\beta_{25-35}$ in cultured hippocampal neurons. MDA was raised by 183% 24 h after the cultures expose to $10\ \mu\text{M}$ $A\beta_{25-35}$, and the activities of SOD and GSH-Px reduced by 39 and 41%, respectively. Pretreatment with α -ZAL or 17β -E2 attenuated the change in MDA, SOD, and GSH-Px. # $P < 0.05$ vs. control, * $P < 0.05$ vs. $A\beta_{25-35}$ group. ($n = 6$)



the substrate for GSH-Px to scavenge peroxides. Notably, decreased tissue GSH-Px concentration was associated with cell damage, moreover, GSH-Px depletion increased the susceptibility of cells to stress-induced cell death [30]. In our experiments, $A\beta_{25-35}$ reduced the activities of SOD and GSH-Px in hippocampal neurons, when cells in the presence of $A\beta_{25-35}$ were pre-incubated with α -ZAL, an elevation in activities of SOD and GSH-Px as well as the enhanced cell survival were observed compared with no α -ZAL treatment, suggesting that α -ZAL significantly attenuated $A\beta_{25-35}$ caused cell damage and cellular redox disequilibrium. Our results showed that α -ZAL protected neurons against $A\beta_{25-35}$ insult through an antioxidant pathway. The effects of α -ZAL on cell viability exhibited a dose-response “plateau” using concentrations ranging from 10^{-9} to 10^{-6} M, thus, higher concentration maybe not more useful for cells.

Our research shows that α -ZAL possesses the ability against oxidative stress induced by $A\beta_{25-35}$ at the concentration 10^{-8} – 10^{-6} M just like as 17β -E2. However, neuroprotective derived from α -ZAL has not been found at

the concentration of 10^{-9} M, although 17β -E2 owns the ability at same concentration. It has been reported that phytoestrogen interact only weakly with known estrogen receptors, providing for neuroprotective efficacy [31], may probably explain α -ZAL's efficiency less than 17β -E2, but in the range of 10^{-8} – 10^{-6} M, the cytoprotection of α -ZAL and 17β -E2 resemble each other. For these reasons, α -ZAL could be a useful neuroprotective agent that mitigates the oxidative stress.

Our study showed that phytoestrogen α -ZAL effectively abates neuron damage induced by $A\beta_{25-35}$, the potential effectiveness of α -ZAL may partly result from its ability to attenuate $A\beta_{25-35}$ -mediated oxidative damage. The present results suggest that α -ZAL may possess potential benefits in prevention of neurodegenerative diseases in postmenopausal women. During the last 10 years, the use of estrogen in postmenopausal women has drawn much attention, especially on their oncogenicity. Future research on α -ZAL should be focused on potential adverse effects and their molecular mechanism of action, these approaches are essential to α -ZAL as a safe alternative for estrogen.

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