Phytoestrogen alpha-zearalanol attenuate endoplasmic reticulum stress to against cultured rat hippocampal neurons apoptotic death induced by amyloid beta25–35

Yilong DONG 1, Ruiqian Li 2, Aimei JIANG 3, Zhong XU 3, Yanmei WANG 3

1 School of Medicine, Yunnan University, Kunming, Yunnan, People's Republic of China
2 The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China
3 The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China

Correspondence to: Yanmei Wang, MD.
The First Affiliated Hospital of Kunming Medical University
295 Xichang Road, Kunming, Yunnan, 650031, People's Republic of China.
tel: +86-871-65324888-2428; e-mail: wangym_kmmu@126.com

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Abstract

OBJECTIVE: Our previous studies demonstrated both phytoestrogen α-zearalanol (α-ZAL) and estrogen is effective decrease Alzheimer's disease (AD)-like apoptotic neuron death, but α-ZAL showed significantly less side-effect on breast and endometrial tissue compared to estrogen, it suggested that α-ZAL can be used as a potential substitute for estrogen. However, the molecular mechanism by which α-ZAL prevents neuron damage remains unclear. Growing evidence suggests that endoplasmic reticulum (ER) stress plays an important role in the process of cell apoptosis in AD; in addition, our published data indicated that α-ZAL possessed the potential ability to stabilize ER function. We therefore hypothesized that ER-stress mechanism maybe involved in the antiapoptotic effect of α-ZAL in this study.

METHODS: Primary rat hippocampal neurons have been cultured and subsequently followed exposed to β-peptide fragment 25–35(Aβ25–35) with or without α-ZAL pre-treatment, and then western blot and flow cytometry techniques has been used to evaluate the intracellular calcium balance, ER stress and apoptotic cell death.

RESULTS: The results showed that Aβ25–35 treatment for 24h induced dramatic neuronal apoptosis, accompanied by an increase in calpain2 expression, a marker of intracellular calcium overload. On the other hand, ER stress sensitive hallmarks, glucose-regulated protein 78 (GRP78), double-stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase (PERK) and C/EBP homologous protein-10 (CHOP10) expressions were up-regulated after Aβ25–35 administration. Importantly, α-ZAL pre-treatment effectively attenuated above changes.

CONCLUSION: These results demonstrated that α-ZAL protects cells against AD-like apoptosis and the effects at least partially by attenuating severely ER stress.
INTRODUCTION

Alzheimer's disease (AD) is the most common progressive neurodegenerative disorder in the aging population, which is pathologically characterized by extracellular amyloid-beta (Aβ) deposits, intracellular aggregates of hyperphosphorylated tau, and neurofibrillary tangles (Nisbet et al. 2015). Although the cause of AD is still unknown, postmenopausal depletion of endogenous estrogens is considered as a significant risk factor for onset of AD in women; therefore, estrogen has been used as an efficacious medicine to reduce the incidence of AD in postmenopausal women (Cander-...). In our previous study, we have reported that α-zearalanol (α-ZAL), a plant-derived phytoestrogen, may effectively maintain serum estrogen concentration in ovariectomized animals but showed less adverse effects on endometrial tissue compared to estrogen (Dong et al. 2006). Meanwhile, Deng and colleagues found that α-ZAL decreased the expression of c-myc, the most commonly amplified oncogenes in human breast cancer, but estrogen increased it in breast tissue, which means α-ZAL was safer than estrogen on breast (Deng et al. 2010). Subsequently, our study confirmed that α-ZAL treatment protected cells against apoptosis induced by Aβ as it can be seen after estrogen treatment (Dong et al. 2011; Dong et al. 2015). Our previous results (Dong et al. 2006; Dong et al. 2011; Dong et al. 2015) in conjunction with the other’s findings (Deng et al. 2010), suggest that α-ZAL may be used as a safe substitute of estrogen for AD prevention in postmenopausal women. Although the protective role of α-ZAL in AD has been supported, the molecular mechanism of how α-ZAL prevents cell death remains to be fully elucidated.

In the eukaryotic cell, endoplasmic reticulum (ER) is an important organelle responsible for protein synthesis, correction of protein folding, post-translation modification and transport. Perturbation of ER functions by accumulation of unfolded proteins, excessive amount of reactive oxygen species (ROS) or changes in intracellular calcium homeostasis triggering an evolutionarily conserved response, termed ER stress (Lindholm et al. 2006). In general, cells respond to ER stress by increasing transcription of ER chaperones, especially 78-kDa glucose-regulated protein (GRP78), and then activated multiple signaling such as double-stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase (PERK) pathway, which facilitates proper protein folding or elimination of misfolded proteins. Moderate ER stress can relieve cellular dysfunction and enhance the chance for survival. However, when stress is severe and prolonged, ER stress itself can induce cell apoptotic death via transcriptional induction genes such as C/EBP homologous protein-10 (CHOP, also known as GADD153) (Tabas & Ron, 2011).

Increasing studies have shown ER stress to be implicated in AD. For instance, increased levels of GRP78 were observed in the hippocampus of AD brains (Hoozemans et al. 2009), and PERK over activation has been suggested as a major neuronal degradation pathway in AD animal (O’Connor et al. 2008). Besides, neuronal apoptotic death can be attenuated when silencing CHOP gene in AD animal (Prasanthi et al. 2011), indicating that improvement of severe ER stress shall be a protective factor of AD. Meaningfully, our published data found that α-ZAL possessed the ability to regulate the Ca²⁺ homeostasis and inhibit the generation of ROS (Dong et al. 2006; Dong et al. 2007). It means that α-ZAL removing the factors which may induce ER dysfunction and potentially attenuating severe ER stress. Therefore, we hypothesized that ER-stress mechanism maybe involved in the advantages of α-ZAL in the present study. To demonstrate the hypothesis, cell samples were collected from Aβ treated primary cultured rat hippocampal neurons with or without α-ZAL pretreated, the expression of parvalbumin and calretinin (the calcium-binding protein benefit to keep intracellular Ca²⁺ balance), calpain2 (a marker of intracellular calcium overload) has been measured, ER stress and apoptosis also has been evaluated to analyse the signaling process involved in neuroprotection of α-ZAL.

MATERIAL & METHODS

Materials

α-ZAL was a gift from Prof. Pingping Zuo at Perking Union Medical College. Aβ25-35, DNAase1 and propidium iodide (PI) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Neurobasal medium, B27 supplement and trypsin was obtained from Invitrogen Corporation (Carlsbad, CA, USA). The protease inhibitor mixture, BCA Protein Assay Kit and enhanced chemiluminescence substrate Kit was obtained from Pierce Biotechnology (Rockford, IL, USA). RIPA lysis buffer was purchased from Beyotime Biotechnology (Shang-
and without α-ZAL pretreatment. Aβ25–35 peptide was dissolved in DMSO and added to the medium by bath application with the final concentration. Cells were incubated for 12h with 10 –7 M α-ZAL, and then exposed to 10 μM Aβ 25–35 for another 24 h. All treatments were performed after 7 days of culture. Cells were incubated for 12h with 10–7 M α-ZAL, and then exposed to 10μM Aβ25–35 for another 24h. α-ZAL was dissolved in DMSO and added to the medium by bath application with the final concentration of DMSO ≤0.1%. Control cells received an equal amount of DMSO, while model cells just receive Aβ25–35 and without α-ZAL pretreatment. Aβ25–35 peptide was dissolved in phosphate buffer saline (PBS), and then incubated for 72 h at 37°C in order to induce aggregation. The aggregated Aβ25–35 was then stored at –80°C until use (Dong et al. 2011).

**Western blot**

Cells were dissociated by trypsin and collected by centrifuge. The cell pellet was washed three times with cold PBS, and then lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. Cell lysates were centrifuged at 12000 rpm at 4°C for 10 min. The supernatants were used for SDS-PAGE after quantification the amount of total protein by using BCA Protein Assay kit. After separated, proteins were transferred on to PVDF membrane and blocked with 5% non-fat milk for 2h at room temperature and then overnight incubation at 4°C with primary antibodies: anti-parvalbumin, anti-calretinin, anti-calpain2, anti-PERK, anti-GRP78 (1:2000), anti-CHOP, and anti-GAPDH (1:5000). All the antibodies were diluted at 1:1000 except the special statement. After washing, the membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1h at room temperature. Immunoreactive bands were detected with the enhanced chemiluminescence. Band intensities were quantified using Image-Pro Express 6.0 software (Media Cybernetics Inc. Rockville, MD, USA).

**Flow cytometry assay using Annexin V/PI staining**

Apoptotic cell death was quantified by flow cytometry using an AnnexinV-FITC kit, according to the manufacturer’s protocol. Briefly, cells were harvested by trypsinization and pelleted by centrifugation. After wash twice with cold PBS, the pellets were re-suspended in 200μl binding buffer, and then incubated with 5μl Annexin V-FITC and 5μl PI at room temperature in the dark. Samples were analyzed using FACScalibur flow cytometer (BD Biosciences, San Diego, CA, USA) using software supplied with the instrument. This allows discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained with Annexin V only) and late apoptotic cells (stained with both annexin V and PI). The samples were tested in six duplicates.
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Several proteins were analyzed to understand the effects of Aβ and α-ZAL on neuronal death. **Fig. 1 A.** α-ZAL pretreatment alleviated intracellular Ca²⁺ overload and the parvalbumin and calretinin decline induced by Aβ₂₅₋₃₅. **B.** Quantitative analysis of protein levels by densitometry. The data from western blot were normalized by taking the value of control group as 1. **p<0.01 vs. control group; #p<0.01 vs. Aβ group. Data are representative of six independent experiments.

**Fig. 2 A.** α-ZAL pretreatment attenuated up-regulated GRP78, PERK and CHOP induced by Aβ₂₅₋₃₅. **B.** Quantitative analysis of protein levels by densitometry. The data from western blot were normalized by taking the value of control group as 1. **p<0.01 vs. control group; #p<0.01 vs. Aβ group. Data are representative of six independent experiments.

Significantly attenuated while cells pretreatment with α-ZAL (p<0.01, compare to Aβ group).

α-ZAL attenuated the elevation of CHOP expression and apoptotic cell death

As suggested by western analysis (Figure 2), Aβ₂₅₋₃₅ alone significantly up-regulated CHOP expression (p<0.01, compare to control group), and the up-regulation of CHOP expression was attenuated by α-ZAL pretreatment (p<0.01, compare to Aβ group). Consistent with CHOP changes, flow cytometry assay demonstrated the apoptotic rate was sharply increased from 2.63% in control cells to 25.34% in cells received Aβ₂₅₋₃₅ alone, but pretreatment with α-ZAL significantly attenuated apoptosis induced by Aβ₂₅₋₃₅ which was shown by decrease of the apoptotic rate to 12.12% (Figure 3).

**DISCUSSION**

Apoptotic neuronal death induced by Aβ is one of the pathological features of AD, although most studies suggested a role of mitochondria signaling in apoptosis, increasing evidences supported that ER stress is involved in the neurotoxicity of Aβ (Thummayot et al. 2016).
2016; Zhou et al. 2016; Kong & Ba 2012). In the present study, we demonstrated that Aβ triggered intracellular calcium overload followed by ER stress, activated ER-related apoptosis signaling to induce apoptotic cell death. However, α-ZAL pretreatment alleviates calcium overload and ER stress induced by Aβ, thereby providing beneficial effects on anti-apoptosis.

Aβ is a soluble protein 40–42 amino acid residue in length which has been found to play a key role in AD. The predominant forms of Aβ in the human brain are Aβ (1–40) and Aβ (1–42), but Aβ (25–35) fragment, is the most toxic region and significantly contributes to the initiation of neurodegenerative changes in AD and is broadly used in research (Millucci et al. 2010).

Accumulating evidence shows that a rise of intracellular Ca²⁺ and its activated downstream signaling pathways is responsible for Aβ二十五三五-induced neuronal death (Kim et al. 2015; Wu et al. 2015). We monitored the change of intracellular Ca²⁺. Although the concentration of cytoplasm Ca²⁺ has not been evaluated directly in the present study, the increase in the expression of calpain2 has been found when cells received Aβ. Calpain2 is a calcium-dependent protein and its expression only can be up-regulated by millimolar calcium level in cytoplasm (Nguyen & Chen 2014). Under physiology condition, cytoplasm calcium concentration maintained at nanomolar level, when the cytoplasm Ca²⁺ increased sharply, the excess Ca²⁺ may trigger calpain2 expression. In the present study, we found that calpain2 expression increased following Aβ treatment, which suggested that there was a non-physiological Ca²⁺ accumulation in the cytoplasm. However, when cells in the presence of Aβ were pretreatment with α-ZAL, the low calpain2 were observed compared with no α-ZAL treatment, suggesting that α-ZAL significantly improved Ca²⁺ overload induced by Aβ. In addition, cells treated with Aβ showed a significantly decline in expression of parvalbumin and calretinin, the calcium-binding protein has been implicated in keeping Ca²⁺ homeostasis in cytoplasm. The down-regulation of parvalbumin and calretinin also has been found in AD animals (Popović et al. 2008), and this decrease may be a major contributing factor to the alternations in Ca²⁺ homeostasis. Our present findings indicated that...
the decrease of parvalbumin and calretinin has been attenuated by α-ZAL. Thus, it maybe postulated that an initial increase in intracellular Ca\(^{2+}\) in neurons following Aβ\(_{25–35}\) exposure, and α-ZAL may improve Ca\(^{2+}\) overload through up-regulate calcium-binding protein.

As a result of intracellular Ca\(^{2+}\) overload, ER stress can be activated. We then evaluated the expression of GRP78. GRP78 is a chaperon protein belonging to the HSP70 family and predominantly resides in the lumen of the ER. Under physiological conditions, GRP78 bind to PERK, activating transcription factor 6 (ATF6) and inositol-requiring kinase 1 (IRE1), which keeps them inactive. During ER stress, GRP78 releases these receptors leading to their activation (Bertolotti et al. 2000). Therefore, GRP78 has been considered as a gatekeeper of the ER response and the unregulated GRP78 is often studied as a biomarker of ER stress (Lee 2005). Similar to Zhou's findings (Zhou et al. 2016), the present study showed the up-regulation of GRP78 when cells treated with Aβ, the elevation of the sensitive ER stress hallmarks suggested that an ER stress occurred in these cells. Meanwhile, our research shows that α-ZAL pretreatment significantly reduced the expression of GRP78, which demonstrates that the excessive ER stress triggered by Aβ is attenuated by α-ZAL.

As stated early, moderate ER stress can relieve cellular dysfunction. However, if the ER stress is severe or prolonged, apoptotic cell death can be induced, which is mainly mediated by PERK signaling (Hetz 2012). PERK activation leads to the phosphorylation of eukaryotic translation-initiation factor 2α (eIF2α) and selectively induces ATF4, which both enhance the expression of CHOP and subsequent mediated apoptosis (Tabas & Ron, 2011). The increased expression of PERK and CHOP accompanied with high apoptotic rate has been observed in cells received Aβ in the present study. Pretreatment with α-ZAL was found to significantly decrease the expression of these proteins and alleviated the apoptotic cell death induced by Aβ. Therefore, this study suggests that α-ZAL offered a protective effect on apoptosis against Aβ assault and attenuated excessive ER stress play a role in these benefits.

Furthermore, it is need to point out, our previews study have compared the efficiency of different α-ZAL concentration on cell viability and found that low concentration (10^-9 M) α-ZAL could not provide protective effect on cells, and there was no significant difference in protective of α-ZAL between medium and high concentration (10^-7 and 10^-6 M) (Dong et al. 2007), this is why we just chose one α-ZAL concentration in the present study. We also found that α-ZAL owns the ability to interact with estrogen receptor (Dong et al. 2015), so we can speculate that α-ZAL may regulate ER-related protein expression through estrogen receptor pathway, further research is necessary to determine this conjecture.

Although there were limits in this work, for example, the interaction between ER and mitochondria in the protective of α-ZAL need to evaluate. Our present results supported that Aβ treated hippocampal neurons undergo apoptosis and α-ZAL exerts anti-apoptotic effects at least in part, attributable to its property neutralize calcium overload and subsequently excessive ER stress. Thus, the protective effects of α-ZAL against AD-associated neurotoxins may help to provide the pharmacological basis of its future clinical usage in the prevention of this neurodegenerative disease especially in postmenopausal women.

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Competing Interests: The authors declare that they have no competing interests.

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α-ZAL attenuate ER stress