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EGb761 protects against $A\beta_{1-42}$ oligomer-induced cell damage via endoplasmic reticulum stress activation andHsp70 protein expression increase in SH-SY5Y cells

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ABSTRACT

Studies have shown that misfolded proteins and endoplasmic reticulum (ER) stress play pivotal roles in the progression of Alzheimer's disease (AD). It has also been reported that ER stress is considered to be a common mediator of apoptosis in neurodegenerative disorders like AD. However, the precise mechanisms leading to neuronal cell death caused by ER stress in AD remain unclear. Hsp70, the major inducible form of the heat shock protein family, functions at the level of chaperone-mediated protein folding. Enhanced expression of Hsp70 suppresses the neurotoxicity caused by protein misfolding. EGb761, an accepted traditional Chinese medicine used to treat AD, was used here to examine the molecular mechanism underlying its protective effect on ER stress and Hsp70. Our study shows that pretreatment with EGb761 overcomes the neurotoxicity of the $A\beta_{1-42}$ oligomer by increasing Hsp70, Grp78, IRE1α and pAkt expression in a dose-dependent manner and significantly decreases cell apoptosis-related protein expression. Our findings suggest that the neuroprotective effect of EGb761 is related to ER stress activation and increased Hsp70 expression, and subsequent activation of Akt. However, the effect of EGb761 on these processes is not direct.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease that is characterized by amyloid-β (Aβ) deposition in the brain (Karran et al., 2011; Selkoe, 1989). Aβ is generated from the sequential cleavage of amyloid precursor protein (APP) by two enzymes, β-secretase and γ-secretase. The prevailing hypothesis called the "amyloid cascade hypothesis" indicates that Aβ aggregation is the initiating mechanistic event and Aβ acts via several pathways to induce synaptic loss and neurodegeneration (Estus et al., 2002; Gilbert, 2013; Luan et al., 2013). Although the mechanisms underlying Aβ-mediated neurotoxicity remain elusive, misfolded proteins and endoplasmic reticulum (ER) stress are recognized as major contributors (Fonseca et al., 2013; Huang et al., 2014; Kohler et al., 2014). The ER is an organelle involved in the folding and processing of proteins in the secretory pathway and in Ca^{2+} homeostasis. Physiological and pathological stimulation such as hypoxia, ischemia and poison, induce ER stress, which is characterized by overexpression of ER molecular chaperones such as Glucose-regulated protein 78 (Grp78), and the unfolded protein response (UPR). These responses help misfolded proteins refold or degrade. Grp78 is the sole ER homologue of heat shock protein 70 (Hsp70) and participates in protein folding and assembly, translocation of protein across the ER membrane, targeting misfolded proteins for degradation and in controlling ER calcium stores (Hendershot, 2004). Abnormal accumulation of proteins in the ER, such as aggregated Aβ cause Grp78 to be released, allowing activation of the UPR, which aids the recovery of proteostasis in the ER lumen. However, if the stress is prolonged or becomes excessive, apoptosis ensues. Furthermore, ER

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stress is considered to be a common mediator of apoptosis in neurodegenerative disorders (Gorbatyuk and Gorbatyuk, 2013). Though many studies have indicated a relationship between ER stress and AD, the precise mechanisms leading to neuronal cell death caused by ER stress in AD remain unclear (Li et al., 2015).

Heat shock proteins (Hsps) are a class of molecular chaperones that facilitate the folding of other proteins and ensuring maintenance of their native conformations under stress (Morimoto and Santoro, 1998; Muchowski and Wacker, 2005). Hsp70 proteins, the major inducible form of the Hsps family, are mainly localized in the cytoplasm, ER and mitochondria (Frydman, 2001) and function at the level of chaperone-mediated protein folding. The evidence available to date suggests that Hsp70 functions in a complex neuroprotective system (Lu et al., 2014; Park et al., 2014) and that enhanced expression of Hsp70 suppresses the neurotoxicity caused by protein misfolding (Bobkova et al., 2014; Paul and Mahanta, 2014; Tsai et al., 2013). Tan et al. found that Grp78, the sole ER homolog of Hsp70, was co-expressed with the Hsp70–Hsp90 chaperone complex (Tan et al., 2011), which can help protein folding and assembly, ER-related protein degradation, calcium binding and regulation of transmembrane ER stress inducers (Whitesell and Lindquist, 2005).

The extract of *Ginkgo biloba* leaves (EGb761), a traditional Chinese medicine, can protect neuronal cells and enhance memory in animals and humans (Andrieu et al., 2008; Luo et al., 2002; Wan et al., 2014). The mechanism of AD has not yet been determined and there are no current efficacious means of AD prevention and treatment. Since EGb761 has antioxidant properties (Seif-El-Nasr and El-Fattah, 1995) and has potential therapeutic efficacy in AD, we investigated the possible neuroprotective effects of EGb761 in an in vitro cell model. Our hypothesis was that activation of ER stress and subsequent co-expression of Hsp70 protein play a key role in the neuroprotective effects of EGb761. We further investigated the relationship between ER stress and Akt activation in this cell model to clarify the role of ER stress in AD.

2. Materials and methods

2.1. Reagents and antibodies

Lyophilized human $A\beta_{42\text{-}1}$ and $A\beta_{1\text{-}42}$, purified by HPLC, was purchased from GL Biochem (Shanghai, China). EGb761 powder, a standardized *G. biloba* extract that contains two major active constituents, 24% flavonol glycosides and 6% terpene trilactones, was purchased from Dr. Willmar Schwabe (Karlsruhe, Germany). The rabbit anti-Hsp70, anti-Grp78, anti-pAkt, anti-caspase-3, anti-caspase-9, anti-caspase-12, anti-IRE1α and mouse anti-β-actin antibodies were purchased from Cell Signaling Technology (MA, USA), and rabbit anti-Akt1 was purchased from Millipore (MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (CA, USA). LY294002, an inhibitor of Akt pathway, was purchased from Cell Signaling Technology (MA, USA). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Beyotime (Shanghai, China).

2.2. Preparation of reagents

Lyophilized human $A\beta_{1\text{-}42}$ was used to prepare $A\beta_{1\text{-}42}$ oligomer as described previously (Dahlgren et al., 2002; Li et al., 2011). EGb761 was dissolved in DMSO at a concentration of 200 mg/mL as stock solution at room temperature. The required concentrations of EGb761 were prepared from the concentrated stock solution diluted in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) cell culture medium.

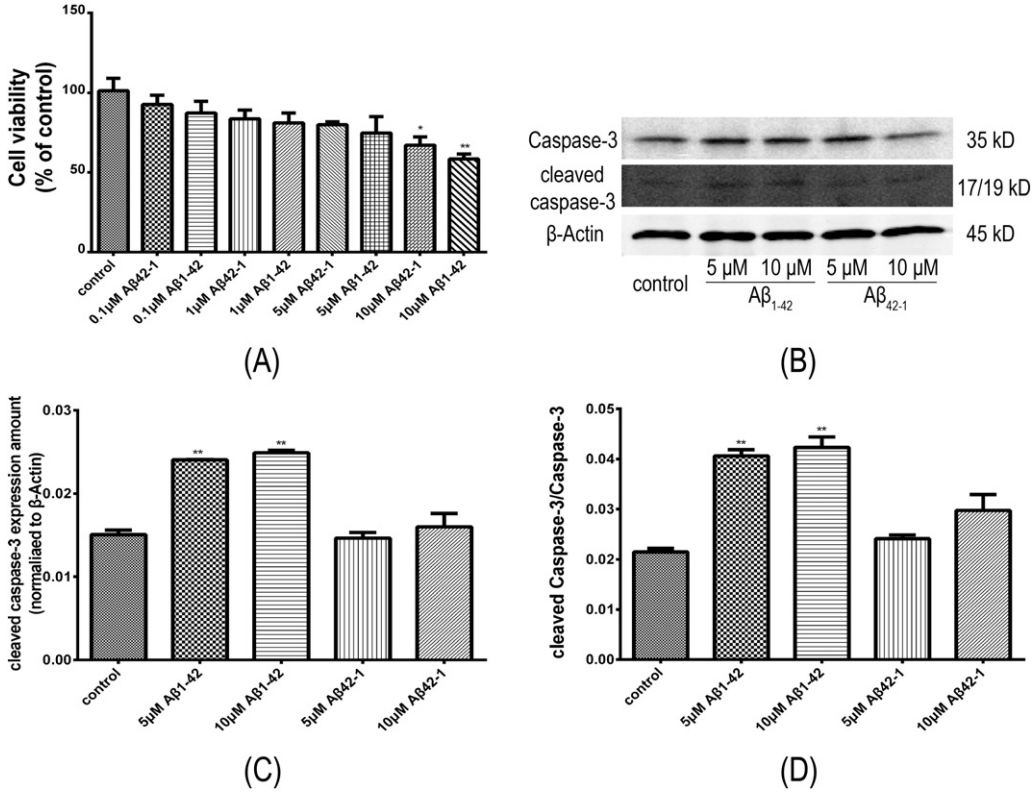

Fig. 1. $A\beta_{1\text{-}42}$ showed more efficient neurotoxicity than its same length reverse peptide $A\beta_{42\text{-}1}$ in SH-SY5Y cells. Cells were treated with different concentrations of $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$ for 24 h. Subsequently, cell viability was measured by the MTT assay. The number of analyzed samples is 6 in each group. (A). In (B), (C) and (D), caspase-3 expression was detected by the Western blotting method. (C) shows the amount of cleaved caspase-3 relative to β-actin, whereas (D) shows the ratio of cleaved caspase-3 to intact caspase-3. The results are shown as mean ± SEM, and each experiment was repeated 3 times (*$p < 0.05$, and **$p < 0.01$, Aβ vs control).

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