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$A\beta_{1\text{-}42}$ induces cell damage via RAGE-dependent endoplasmic reticulum stress in bEnd.3 cells

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ABSTRACT

Blood-brain barrier (BBB) breakdown has been determined to play a critical role in the pathogenesis of Alzheimer's disease (AD). However, the underlying mechanisms of BBB disruption in AD remain unclear. Our previous study suggested that the receptor for advanced glycation end-products (RAGE) functioned as a signal transduction receptor in $A\beta_{1-42}$-induced damage in endothelial cells. In our present study, we revealed that RAGE-mediated endoplasmic reticulum stress (ERS) is essential for Aβ-induced endothelial cell damage. Here, we found that $A\beta_{1-42}$ activated ERS by upregulation of Grp78, xbp-1 and CHOP in endothelial cells and that $A\beta_{1-42}$-resulted lesions, including the upregulations of caspase-12 and caspase-3, the augment of bax/bcl-2 ratio, and the downregulations of ZO-1 and Occludin in bEnd.3 cells, were ameliorated by the pretreatment of salubrinal, an ERS inhibitor. Furthermore, the expressions of Grp78, xbp-1 and CHOP induced by $A\beta_{1-42}$ were blocked by transfection of RAGE small interfering RNA (siRNA), which indicated that $A\beta_{1-42}$ activated ERS in a RAGE-dependent manner. Additionally, bEnd.3 cells transfected with RAGE siRNA showed lower expressions of caspase-12 and caspase-3, decreased bax/bcl-2 ratio, and higher expressions of ZO-1 and Occludin following Aβ1-42 treatment, comparing to control cells. In conclusion, our data demonstrated that $A\beta_{1-42}$ induced endothelial cells damage via activation of ERS in a RAGE-dependent manner.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorders leading to senile dementia [1] and is one of the important causes of death in the elderly [2]. The pathogenic mechanism of AD is complex and has not been revealed to date. "Amyloid Cascade Hypothesis" proposed that the accumulation of amyloid-beta (Aβ) in brain is the main cause for the development of AD [3]. Blood-brain barrier (BBB) is a tightly sealed barrier that regulates substance exchange between peripheral blood and central nervous system (CNS), and is of significance to maintain brain homeostasis [4]. BBB is considered to play an important role in regulating the dynamic balance of Aβ in brain [5]. Dysfunction of brain capillary, which occurs early in AD [9], has been widely described in AD transgenic animals and AD patients [6–8]. Cerebrovascular dysfunction results in impaired clearance of Aβ from brain and increased influx of peripheral Aβ into brain, thus promoting Aβ deposition in brain parenchyma and cerebrovascular, which in turn induces inflammation and dysfunction of brain capillary endothelium, ultimately leads to neurodegeneration in AD [10,11]. Tight junction (TJ) between endothelial cells is one of the critical elements responsible for barrier property of BBB [12]. The abnormalities of TJ scaffold proteins have been reported in many CNS disorders including AD [13,14]. Aβ disrupts TJs thereby compromise the integrity of BBB and disturb brain homeostasis [15], ultimately leading to neurodegeneration. Consistently, A recent study [16] confirms that brain microvascular dysfunction is associated with symptom severity and neurodegeneration in AD, highlighting the significance of cerebrovascular pathology in AD. Therefore, finding the molecular mechanisms which lead to brain capillary disturbance and BBB dysfunction, and seeking effective methods to interfere BBB breakdown may be meaningful to delay neurodegenerative progress in AD.

ERS is one of the mechanisms involved in endothelial cells damage [17,18]. Aβ has been implied to induce cerebrovascular injury via ERS [19]. Previous studies indicated that Aβ Oligomer, but not Aβ fibrillar, induced cell apoptotic death by activating ERS [20]. Data from in vitro and in vivo experiments confirmed that Aβ promotes the expressions of the markers of ERS and increases the level of effectors of ERS-related apoptosis pathway such as CHOP and caspase-12 [21]. Prolonged ERS

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leads to accumulation of APP in brain endothelium, enhancing cell death in endothelial cells [22]. In contrast, blockage of ERS is indicated to effectively ameliorate cells damage induced by Aβ [23]. These data demonstrate that ERS is one of the significant molecular mechanisms involved in Aβ cytotocixity. However, the regulators involved in Aβ-induced activation of ERS are unconfirmed

The receptor for advanced glycation end products (RAGE), belonging to members of Immunoglobulin superfamily receptors, ubiquitously expresses in variety kinds of cell types and tissues, and exerts as a transmembrane cell-signaling receptor [24]. Physiologically, RAGE has much lower expression in brain while its expression is found to substantially increase in AD brain [24,25]. Evidences from numerous studies demonstrate that RAGE is a critical receptor mediating Aβ cytotoxicity, thus plays a pivotal role in the development of AD pathology [26,27]. At the BBB, on the one hand, RAGE functions as a transporter which mediates Aβ influx into brain parenchyma and subsequently results in Aβ deposition; on the other hand, RAGE interacts with Aβ and triggers a cascade of reactions, such as oxidative stress and inflammatory response, which lead to neurons loss and cerebrovascular damage [28]. Studies show that ERS activated by RAGE correlates with multiple pathogenic responses, while blockage of RAGE signaling is effective to restrain activation of ERS and alleviates cells damage and apoptosis mediated by RAGE [29–31]. However, the role of RAGE in Aβ-induced ERS has not been addressed yet in endothelial cells.

Our previous data implicated the role of ERS in induction of neurons apoptosis by Aβ [32]. We also confirmed that RAGE function as an important mediator in Aβ-induced endothelial cells damage and alteration of TJ scaffold protein in vitro BBB model [33]. Based on data above, we speculate that Aβ activates ERS in a RAGE-dependent manner in brain endothelial cells and subsequently leads to disorder of endothelial cells.

2. Materials and methods

2.1. Cell culture and treatments

The bEnd.3 cells was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μM streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured every 2–3 days. For experiments, cells were grown to 70–80% confluence and the media were replaced with Opti-MEM (Invitrogen).

2.2. Reagents and antibodies

Lyophilized human $A\beta_{1-42}$ was purchased from Chinapeptides (Shanghai, China). Dimethylsulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)−2, 5-diphenyl tetrazolium bromide (MTT) were purchased from sigma(CA,USA), whilst the rabbit anti-Grp78, anti-RAGE, anti-caspase-12, anti-caspase-3, anti-bax, anti-bcl-2 primary antibodies and mouse anti-CHOP and anti-β-actin primary antibodies were purchased from Cell Signaling Technology (MA, USA). The rabbit anti-xbp-1, anti-ZO-1 and anti-Occludin primary antibodies were purchased from Invitrogen (CA, USA). The goat anti-rabbit and anti-mouse secondary antibodies were purchased from LI-COR (CA, USA). Sodium fluorescein (Na-F) powder was purchased from Kayon Bio-tech Co. (Shanghai, China).

2.3. Preparation of $A\beta_{1-42}$ oligomer

$A\beta_{1-42}$ oligomer was prepared as described [34]. In briefly, $A\beta_{1-42}$ peptides were dissolved in dry DMSO in a concentration of 2 mM and stored at −20 ℃. For oligomer preparation, 2 mM $A\beta_{1-42}$ in DMSO was diluted into a concentration of 100 μmol/L in cold Opti-MEM media and incubated at 4 ℃ for 24 h before use.

2.4. Measurement of cell viability

Cells were plated into 96-well plates. After the various treatments, cell viability was determined by the MTT assay. MTT (5 mg/mL) was added to each cell culture well followed by 4 h incubation at 37 °C. The medium was then aspirated and DMSO was added to each culture well to dissolve the formazan crystals. Absorbance was measured at 490 nm using a micro-plate reader (Bio Tek, VT, USA) Cell viability was expressed as a percentage of value relative to the control cells.

2.5. BBB permeability measurements

The endothelial barrier function was investigated by measuring the leakage of Na-F across endothelial cell monolayer, following a previously described protocol with slight modification [33]. In briefly, bEnd.3 cells were plated into polycarbonate membrane Transwell permeable inserts (Corning, MA, USA) and grown to 70–80% confluence followed by different treatment, then Hank's Balanced Salt solution (HBSS) was added into the basolateral compartments and culture medium in the inserts was replaced by HBSS assay buffer containing 10 μg/mL Na-F. After 30 min, the samples of HBSS buffer from the basolateral compartments were measured by Typhoon FLA 9500 (GE Healthcare, USA) at wavelengths of 473 nm (excitation) and 535 nm (emission).

2.6. Analysis of plasma membrane damage via the lactate dehydrogenase release (LDH) assay

Plasma membrane damage was detected by the release of lactate dehydrogenase (LDH) assay. The level of LDH released in the cell culture supernatant was detected by a LDH cytotoxicity assay detection kit (Beyotime, China) following the manufacturer's instructions.

2.7. Knockdown of RAGE with siRNA

RAGE small interfering RNAs (siRNA, sense 5′-GCCAGAAAUU GUGGAUCCUTT-3′, and antisense 5′-AGGAUCCACAAUUU CUGGCTT-3′) were synthesized by Genepharma. Cells were grown to 60–70% confluence and then transfected with Lipofectamine RNAiMAX (Invitrogen) and siRNA according to the manufacturer's instructions. RT-PCR was used to verify the silencing efficiency after 24 h of siRNA transfection.

2.8. Western blot

Cells were collected and lysed for protein expression analysis. Equal amounts of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, protein was transferred onto a nitrocellulose membrane (Millipore) and the membrane was blocked with 5% fat-free milk for 1 h at room temperature. Immunoblots were incubated with primary antibodies overnight at 4 °C. The membranes were washed 3 times with TBST and then incubated with secondary antibody (LI-COR, USA) for 1 h at room temperature. Western blot bands were captured using the Odyssey infrared fluorescence imaging system (LI-COR, USA).

2.9. Statistical analysis

All results are expressed as the Mean ± SD. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). All experiments were repeated three times independently. Statistical significance of difference among different groups was analyzed by one-way ANOVA or Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.