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$A\beta_{1-42}$ oligomer induces alteration of tight junction scaffold proteins via RAGE-mediated autophagy in bEnd.3 cells

Yuanjin Chan[a], Wenjing Chen[a], Wenbin Wan[b], Yanjie Chen[a], Yaming Li[a,*], Chunyan Zhang[c,*]

[a] Geriatrics Department of Chinese Medicine, Huadong Hospital, Fudan University, Shanghai 200040, China
[b] Department of Neurology, Renji Hospital, Shanghai Jiaotong University, Shanghai 200127, China
[c] Department of Chinese Medicine, East Hospital, Tongji University, Shanghai 310000, China

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ABSTRACT

Compelling evidences have shown that amyloid-β (Aβ) peptide is one of the major pathogenic factors resulting in blood-brain barrier (BBB) disruption in Alzheimer's disease (AD). However, the mechanism underlying BBB breakdown remains elusive. In our present study, we employed murine brain capillary endothelial cells (bEnd.3) as an in vitro BBB model to investigate the role of autophagy in $A\beta_{1-42}$ oligo induced BBB disruption. We first identified $A\beta_{1-42}$ oligo cytotoxicity to bEnd.3 cells as observed in the reduced cell viability and downregulation of ZO-1, Occludin and Claudin-5. Based on the observation that both downregulated expression of p-mTOR/m-TOR and upregulated ratio of LC3-II/β-actin were induced by $A\beta_{1-42}$ oligo, we then applied 3-MA, an inhibitor of autophagy, to test the role of autophagy in $A\beta_{1-42}$ oligo induced Tight junction (TJ) proteins damage. Results have shown that 3-MA partially reversed $A\beta_{1-42}$ oligo induced downregulation of ZO-1, Occludin and Claudin-5, which was further determined by LC3 siRNA. We also used rapamycin to activate autophagy and found that TJ proteins damage induced by Aβ1–42 was deteriorated even further. Given that the receptor of advanced glycation end-products (RAGE) is a pivotal receptor that mediates Aβ toxicity, RAGE siRNA was utilized to identify the involvement of RAGE in $A\beta_{1-42}$ oligo induced autophagy. The results demonstrated a suppressed autophagy with increased p-mTOR/m-TOR and decreased LC3-II/β-actin as well as increased ZO-1, Occludin and Claudin-5 in transfected cells after $A\beta_{1-42}$ oligo treatment, as compared to the non-transfected group. In summary, these results suggested that $A\beta_{1-42}$ oligo induced TJ proteins disruption via a RAGE-dependent autophagy pathway.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease characterized by early neurovascular dysfunction, progressive neurodegeneration and several pathogenic mechanisms which include selective loss of neurons, presence of amyloid-β (Aβ) plaques and neurofibrillary tangles in the brain [2]. In particular, "Amyloid Cascade Hypothesis" proposes that the accumulation of Aβ in brain is to be responsible for the development of AD. Blood–brain barrier (BBB), which is composed of the highly specialized central nervous system (CNS) microvascular endothelial cells, was first described by Ehrlich more than 100 years ago and was considered to protect the CNS from infections and toxins [54]. Few studies have indicated that BBB acts as a tightly sealed barrier that regulates substance within the constantly changing milieu in the blood stream [20,54]. Hence, the neurovascular hypothesis of AD proposes that neurovascular dysfunction and disruption contribute to the onset and progression of cognitive decline [19,3,41]. This cerebral microvascular pathology may promote other Aβ mediated pathologies which can subsequently impair vascular clearance of brain Aβ and increase the influx of peripheral Aβ into the brain, thereby accelerating the accumulation of senile plaques formation [14,36]. The tight junction(TJ), consisting of TJ proteins such as zo-1, occludin and claudin-5, is reported to be one of the most important constituents that maintain the barrier property of BBB and restrict the transport of macromolecules across the BBB [30,4]. Moreover, abnormalities of TJ proteins have been reported in many CNS disorders including AD. Most studies demonstrate that Aβ disrupts TJ scaffold proteins leading to the compromise of the integrity of BBB and disturbance of brain homeostasis, ultimately resulting in neurodegeneration [11,32]. In vitro, $A\beta_{1-42}$ was reported to enhance BBB permeability, through reduction of ZO-1, claudin-5 and Occludin expression [43,44,46]. On the other hand, in vivo studies have shown that cerebral amyloid angiopathy is accompanied by a dramatic loss of Occludin, claudin-5, and ZO-1 in Aβ-laden capillaries, indicating that Aβ is closely related to damage of TJ scaffold proteins [10,29,51].

The autophagy-lysosomal system, one of the major protein

* Corresponding authors.
E-mail addresses: Doctorymli@163.com (Y. Li), cyzhang0810@163.com (C. Zhang).

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degradation pathways in eukaryotic cells, is responsible for degrading and recycling cell constituents [21,37]. After being converted into double membrane autophagosomes, the defective organelles, obsolescent cellular organelles and long-lived structural proteins, were further transferred to lysosomes [21,37,53], where they were degraded by lysosomal enzymes. Normally, the autophagosomal-lysosomal pathway (ALP) assures proper control of protein quality and quantity [24]. However, compelling data from different studies about the role of autophagy in homeostasis of Aβ in Alzheimer's disease demonstrate that impaired autophagy is closely related to the Aβ neurotoxicity [28,33,35,7]. Evidences from numerous studies show that autophagy plays an important role in regulating TJ proteins disruption [27,38]. Taken together, these studies suggest that autophagy accounts for disruption of TJ proteins. However, the underlying molecular mechanisms through which autophagy involved in Aβ induced autophagy remain unclear.

The receptor for advanced glycation end products (RAGE) belongs to members of cellular membrane surface immunoglobulin superfamily receptors. These receptors mediate several cell signaling pathways and further participate in the pathological mechanism of multiple CNS disorders including AD [13,15]. In addition to enhancing the accumulation of Aβ in brain parenchyma, RAGE mediates Aβ induced cellular signals, including NF-kB, MAPK, PRAK, which involve in inflammation, apoptosis, and autophagy [25,39,8]. Since our previous study affirmed that RAGE plays an important role in Aβ-induced BBB injury [44], we propose a possible underlying molecular mechanism whereby Aβ induce TJ proteins disruption via RAGE-mediated autophagy. To prove our hypothesis, we applied several autophagy modulators and LC3 SiRNA to determine the involvement of autophagy in Aβ induced BBB damage, and RAGE SiRNA to determine the role of RAGE in the autophagy induced by Aβ.

2. Material and methods

2.1. Reagents and antibodies

Lyophilized human Aβ1–42 purified by HPLC, was purchased from GL Biochem (Shanghai, China). Dimethylsulfoxide, (DMSO), 3-(4,5-Dimethylthiazol- 2-yl) − 2, 5-diphenyl tetrazolium bromide (MTT) were purchased from sigma(CA,USA), while the rabbit anti-LC3 primary antibodies, rabbit anti-RAGE primary antibodies and mouse anti-β-actin primary antibodies were purchased from Cell Signaling Technology (MA, USA). The rabbit anti-ZO-1, anti-Claudin-5 and anti-Occludin primary antibodies were purchased from Invitrogen (CA, USA). The rabbit anti-p-mtor and mtor primary antibodies and protein marker were purchased from Thermo Scientific (USA)

2.2. Cell culture and treatments

The murine mouse brain capillary EC line bEnd.3 were 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). Cultures were maintained at 37 ˚C in a humidified atmosphere containing 5% CO2 and then subcultured every 2–3 days. For all experiments, bEnd.3 cells were growth-arrested at 70–80% confluence without media change and then the media were replaced with Opti-MEM (Invitrogen) before the treatments were applied as described below.

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

$A\beta_{1-42}$ oligomer was prepared as described. $A\beta_{1-42}$ was dissolved in hexafluoroisopropanol giving a concentration of 1 mmol/L and aliquoted into sterile microcentrifuge tubes. Then hexafluoroisopropanol was evaporated in order to get $A\beta_{1-42}$ peptides and the obtained $A\beta_{1-42}$ 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 dry DMSO was diluted into ice-cold Opti-MEM to bring the peptide to a final concentration of 100 μM and incubated at 4 ˚C for 24 h before utilization.

2.4. Measurement of cell viability

Cell viability was measured by using MTT assay. Firstly, bEnd.3 cells were plated into 96-well plates overnight. After individual treatments, 20 μL MTT (5 mg/mL, Sigma) was added to each cell culture well containing 100 μL of medium followed by 4 h incubation at 37 ˚C. The medium was then gently aspirated and formazan crystals were lysed in 100 μL DMSO by gently shaking the plate. Absorbance was measured at 490 nm using a micro-plate reader (BioTek, VT Lab, USA). Cell viability was expressed as a percentage of the value relative to the control cultures

2.5. Knockdown of LC3 with siRNA

RAGE small interfering RNAs (siRNA, sense 50-GCCAGAAAUU GUGGAUCCUTT-30, and antisense 50-AGGAUCCACAAUUU CUGG CTT-30) were synthesized by Genepharma. LC3 small interfering RNAs (siRNA, sense 50-AUAAUCACUGGGAUCUUGGUG-30, and antisense 50-CACCAAGAUCCCAGUGAUUAU-30) were synthesized by Biotend. Cells were grown to 70–80% confluence before transfection of cells with Lipofectamine RNAiMAX (Invitrogen) and siRNA according to the manufacturer's instructions. RT-PCR was used to verify the silencing efficiency following 24 h of siRNA transfection. The cells were then treated with or without $A\beta_{1-42}$- oligomer for another 24 h followed by the qRT-PCR and western blot analysis.

2.6. Immunofluorescence staining

BEnd.3 cells were cultured to confluence on glass cover slip in Opti-MEM (Invitrogen) before the treatments were applied as described below. After that, cells were washed with PBS and fixed in 4% formaldehyde in PBS for 20 min at room temperature, and then were blocked with 5% bovine serum albumin(BSA, diluted in PBS) for 1 h at room temperature. Then, cells were incubated with rabbit anti-ZO-1 and rabbit anti-LC3 primary antibodies overnight at 4 ˚C and washed with PBS three times followed by incubation with goat anti-rabbit IgG 488-conjugated secondary antibody for 1hrs. All samples were assessed using a fluorescence microscope.

2.7. Western blot analysis

Cells were collected at 80–90% confluence and lysed in 90 μL of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM TrisHCl (pH 7.4), 20 mM Na-F, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na 3VO4) with 15 μL of phenylmethylsulfonyl fluoride(PMSF) to yield whole cell extracts for protein expression analysis. Each of protein samples contained (40 μg) were denatured and subjected to 10% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE). After electrophoresis, protein was transferred onto a nitrocellulose membrane (NC). Membranes were blocked with 5% fat-free milk for 3hrs at room temperature, and then incubated with primary antibodies overnight at 4 ˚C. The membranes were washed 3 times with TBST and then incubated with species-specific secondary antibody (LI-COR, USA) for 1 h at room temperature. The images of western blot bands were captured using Odyssey infrared fluorescence imaging system (LI-COR, USA).

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from bEnd.3 cells by using TRIzol reagent (Invitrogen, CA, USA) and a ReverTra Ace qPCR RT kit (TOYOBO) was used to obtain cDNA. For real-time PCR, SYBR Green RT-PCR Master