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Progesterone exerts neuroprotective effects against Aβ-induced neuroinflammation by attenuating ER stress in astrocytes

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ABSTRACT

The deposition of amyloid-β (Aβ) and neuroinflammation are critical pathological features of Alzheimer's disease (AD). Astrocytes are considered the principal immunoregulatory cells in the brain. Neurosteroid progesterone (PG) exerts neuromodulatory properties, particularly its potential therapeutic function in ameliorating AD. However, the role of PG and the neuroprotective mechanism involving in the regulation of neuroinflammation in astrocytes warrant further investigation. In this study, we found that Aβ significantly increased the processing of neuroinflammatory responses in astrocytes. The processing is induced by an increase activity of PERK/eIF2α-dependent endoplasmic reticulum (ER) stress. Additionally, the inhibition of ER stress activation by Salubrinal significantly suppressed the Aβ-induced neuroinflammatory responses in astrocytes. While the treatment of astrocytes with Aβ caused an increase of neuroinflammatory responses, PG significantly inhibited Aβ-induced neuroinflammatory cytokine production by suppressing ER stress activation together with attenuating PERK/eIF2α signalling. Taken together, these results indicate that PG exerts a neuroprotective effect against Aβ-induced neuroinflammatory responses, and significantly suppresses ER stress activation, which is an important mediator of the neurotoxic events occurring in Aβ-induced neuroinflammatory responses in astrocytes. These neuroprotective mechanisms may facilitate the development of therapies to ameliorate AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in a gradual decline in cognitive processes. The neuropathology of this disease is characterized by intracellular neurofibrillary tangles, senile plaques, and excessive neuronal loss [1]. The deposition of amyloid-β (Aβ) has been considered an extremely critical factor for AD development, but the mechanism underlying Aβ-induced neurotoxicity is not fully understood. It was recently recognized that neuroinflammation is a prominent feature in the modulation of AD processing and that a persistent secretion of proinflammatory cytokines is observed [2]. However, the sequence of events leading to neuroinflammation remains unclear, and the mechanism underlying Aβ-induced neuroinflammation remains an area of active investigation.

Astrocytes are the most abundant glial cells in the central nervous system. Astrocytes dynamically endow neurons with trophic support and modulate information processing [3]. However, prolonged and widespread reactive astrocytes are characteristically found in the AD brain. Once activated, astrocytes produce several proinflammatory signal molecules, including cytokines, growth factors, complement molecules, and chemokines [4]. The released cytokines, particularly interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, are the major effectors of neuroinflammatory signals and affect neurophysiologic mechanisms regarding cognition and memory [5]. Indeed, these reactive astrocytes revolve around senile plaques and neurofibrillary tangles in the AD brain, suggesting that Aβ deposition is a potent trigger of astrocytic activation in the AD brain. However, the mechanistic connection between Aβ deposition and astrocytic inflammation is not fully understood.

ER stress has recently been regarded as a vital pathophysiological mechanism in the development of many diseases [6–8] and particularly as a relevant pathological factor of AD [9]. Multiple lines of evidence obtained from studies of post-mortem brain tissue of AD patients or animal models have led to the wide acceptable of a relationship between ER stress and Aβ-induced cytotoxicity [8, 10]. Aβ severely disrupts endoplasmic reticulum (ER) function and causes excessive activation of ER stress in neurons [11]. Excessive and prolonged ER stress activation is detrimental to neurons because a delayed defence decreases the viability of neurons and initiates an apoptotic program inside the cells [12]. Therefore, it is obvious that ER stress may be a crucial factor in the pathogenesis of AD. In addition, ER stress is linked

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with some of the major inflammation and stress signalling networks [13]. That suggests the activation of ER stress may correlate with neuroinflammatory response *via* a potential pathway.

Steroid hormones and their metabolites within the central nervous system (CNS) are commonly defined as neuroactive steroids or neurosteroids [14]. The neuroprotective properties of neurosteroids have been recently reported in numerous studies [15–17]. One particular focus of previous studies is the neurosteroid progesterone (PG) and its neuroprotective capacity in neurodegenerative disorders [18, 19]. Our previous work found that the level of neurosteroids PG in the brain of AD rats abnormally decreased. However, subcutaneous injection of PG significantly improved cognitive abilities of AD rats [20]. Furthermore, we found that PG exhibited a neuroprotective function by inhibiting Aβ-induced mitochondrial apoptosis through mediating JNK signalling pathway in neurons [21]. PG exhibits a potential therapeutic function in the amelioration of AD, but the potential protective mechanism has not been fully elucidated. Particularly, the role of PG and the neuroprotective mechanism involving the regulation of astrocytic function warrant further investigation. In consideration of this issue, a better understanding of PG and its potential as a neuroprotective agent against AD is of great significance.

Therefore, in this study, we aimed to elucidate the molecular mechanism underlying Aβ-induced inflammatory responses in astrocytes and investigate whether PG exerts a protective effect against Aβ-induced neuroinflammation and its potential neuroprotective mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO), poly-*D*-lysine, Salubrinal (Sal), Tunicamycin (TM), and $A\beta_{1-42}$ fragments and progesterone were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). DAPI and trypsin were purchased from Solarbio Inc. (Beijing, China). DMEM, foetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA, USA). Antibodies to phospho(P)-eIF2α, *P*-PERK, total(T)-eIF2α, *T*-PERK, GRP78, GFAP and β-actin were purchased from Cell Signal Technology Inc. (Beverly, MA, USA). Goat *anti*-rabbit lgG/FITC was purchased from Bioss Inc. (Beijing, China).

2.2. Cell culture and treatment

2.2.1. Primary astrocyte culture

Astrocytes were prepared from newborn Sprague–Dawley rat pups (<24 h). The cerebral cortices were removed from brain and digested at 37 °C for 15 min with trypsin (1.25 g/L). The cells were seeded into poly-*L*-lysine-coated coverslips or multiwell plates at either 1×10^5 cells/cm^2 (Immunofluorescence analysis) or 1×10^6 cells/cm^2 (western blotting analysis) in DMEM medium supplemented with 10% FBS, and incubated at 37 °C in a 5% CO_2 incubator. The medium was exchanged every 3 days after the cultures were primarily confluent astrocytes (>90%). The confluent cultures were shaken overnight to remove microglia contamination from adherent astrocytes after 10 days incubation. More than 95% of the incubated cells were astrocytes as identified by immunofluorescent staining for GFAP.

2.2.2. Oligomeric $A\beta_{1-42}$ preparation

$A\beta_{1-42}$ oligomers were prepared according to the methods [22]. An $A\beta_{1-42}$ monomer was prepared by evaporating 2 mg $A\beta_{1-42}$ dissolved in 1, 1, 1, 3, 3, 3, hexafluoro-2-propanol at room temperature for 30 min with N2 gas. The monomer was then dissolved in DMSO and diluted to 10 μM with Dulbecco's Modified Eagle's Media (DMEM, Gibco). The diluted solution was then incubated at 37 °C for 24 h to form oligomers. The $A\beta_{1-42}$ oligomers preparations were centrifuged at 16,000*g* to remove any insoluble fibrils, and next, the supernatant was diluted in DMEM prior to addition to cultures at the final concentrations.

2.3. Immunofluorescence assay

The cultured primary astrocytes were cultured with standard conditions described above (Methods-2.2.1), and then exposed to $A\beta_{1-42}$ oligomers (Methods-2.2.2) for indicated times. The cells were washed with ice-cold PBS and fixed in 4% ice-cold paraformaldehyde for 30 min. Fixed cells were permeabilized with 0.05% Triton X-100, and blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Next, the cells were incubated with primary antibody (*anti*-GFAP, 1:300 dilution) overnight at 4 °C in a humidified container. The next day, the cells were washed and then stained with FITC-conjugated goat *anti*-rat lgG secondary antibodies (1:100 dilution) for another 1 h. After washed with PBS, nuclei were subsequently stained with DAPI for 5 min. Images were visualized under fluorescence microscope (IX-81, Olympus).

2.4. Western blotting assay

The cells were lysed, and proteins were extracted from cultured primary astrocytes using RIPA Lysis buffer (Beyotime Institute of Biotechnology, Jiangsu China). The supernatants were collected and total proteins were measured using a BCA protein assay kit. The total extracted proteins were separated on 10% or 15% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA). Membranes were blocked with 5% non-fat milk in tris-buffered saline containing 0.1% Tween for 2 h at room temperature and incubated with primary antibody (*anti*-GRP78, *P*-eIF2α, *P*-PERK, *T*-eIF2α, *T*-PERK and β-actin 1:1000) at 4 °C overnight. After washing with TBS-T (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20), membranes were incubated with secondary antibody for 2 h at room temperature. Blots were developed with an ECL detection kit, and integrated densities of bands were measured by Image J software.

2.5. ELISA assay

Astrocytes treatment supernatants were collected and the levels of IL-1β and TNF-α in culture supernatant were determined by ELISA according to the manufacturer's instructions. Briefly, the supernatants (100 μL) were added to the appropriate well in rat *anti*-IL-1β and TNF-α pre-coated plates. Next, 50 μL biotin-labelled *anti*-IL-1β and TNF-α were added to each well. After 2 h incubation, the plates were washed 5 times with wash buffer, and 50 μL substrate A was added into each well and incubated for 1 h at 37 °C. In a following step, 50 μL substrate B was added, and the plates were kept in the dark at 37 °C for 30 min. Finally, 50 μL stop solution was added to terminate reaction. Detection and quantification of the plates were carried out with a microplate reader (Molecular Device Corporation, Sunnyvale, CA, USA) at 450 nm. The results were expressed as pg/mL for culture media.

2.6. Statistical analysis

The experimental results were expressed as the mean ± SD. Statistically significant difference analysis was carried out by student's *t*-test and one-way ANOVA followed by the Dunnett's test with SPSS 13.0 software. Mean values were considered to be statistically significant in this study at $p < 0.05$ or less.

3. Results

3.1. Aβ upregulates GFAP expression and induces morphological changes in astrocytes

Astrocytes are susceptible to activate in a variety of neuropathological states [23]. The upregulation of GFAP expression and morphological