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Aβ-Induced Drp1 phosphorylation through Akt activation promotes excessive mitochondrial fission leading to neuronal apoptosis

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ABSTRACT

Mitochondrial dysfunction is known as one of causative factors in Alzheimer's disease (AD), inducing neuronal cell death. Mitochondria regulate their functions through changing their morphology. The present work was undertaken to investigate whether Amyloid β (Aβ) affects mitochondrial morphology in neuronal cells to induce apoptosis. Aβ treatment induced not only the fragmentation of mitochondria but also neuronal apoptosis in association with an increase in caspase-9 and -3 activity. Calcium influx induced by Aβ up-regulated the activation of Akt through CaMKII resulting in changes to the phosphorylation level of Drp1 in a time-dependent manner. Translocation of Drp1 from the cytosol to mitochondria was blocked by CB-124005 (an Akt inhibitor). Recruitment of Drp1 to mitochondria led to ROS generation and mitochondrial fission, accompanied by dysfunction of mitochondria such as loss of membrane potential and ATP production. ROS generation and mitochondrial dysfunction by Aβ were attenuated when treated with Mdivi-1, a selective Drp1 inhibitor. Furthermore, the sustained Akt activation induced not only the fragmentation of mitochondria but also the activation of mTOR, eventually suppressing autophagy. Inhibition of autophagic clearance of Aβ led to increased ROS levels and aggravating mitochondrial defects, which were blocked by Rapamycin (an mTOR inhibitor). In conclusion, sustained phosphorylation of Akt by Aβ directly activates Drp1 and inhibits autophagy through the mTOR pathway. Together, these changes elicit abundant mitochondrial fragmentation resulting in ROS-mediated neuronal apoptosis.

1. Introduction

Alzheimer's disease (AD), characterized by memory deficits and cognitive impairments, is one of the most common types of age-related brain disorders causing dementia. Amyloid β (Aβ) has been considered as a major risk factor of AD, due to it having high toxicity toward neuronal function; eliciting synaptic hyperexcitability and neuronal cell loss *via* oxidative damage [1–3]. Aβ leads to neuronal cell death which is one of the major hallmarks of AD, along with abnormal mitochondria, neurofibrillary tangles (NFTs) and amyloid plaques [4,5]. Accumulation of dysfunctional mitochondria is frequently observed in the brains of AD patients [6,7]. Neuronal cells require much energy and are reliant on proper mitochondrial function. Many studies have focused on mitochondria as a significant controller in neurodegenerative diseases due to their regulatory role in cell fate and energy generation. Accordingly, the importance of preserving mitochondrial function has been emerged as a therapeutic target in the pathogenesis of AD [8,9].

The function of mitochondria is tightly related to their morphological transition between fusion and fission. An imbalance of mitochondrial dynamics causes the accumulation of fragmented mitochondria and

Abbreviations: Aβ, Amyloid β; AD, Alzheimer's disease; Akt, protein kinase B; AraC, cytosine arabinoside; ATP, adenosine triphosphate; CaM, calmodulin; CaMKIα, Ca^{2+}/calmodulin-dependent kinase type 1 alpha; CaMKII, Ca^{2+}/calmodulin-dependent kinase type II; CDK, cyclin dependent kinase; DMEM, Dulbecco Modified Eagle Medium; Drp1, dynamin-related protein; ERK, extracellular signal-regulated kinase; ETC, electron transport chain; FBS, fetal bovine serum; Fis1, mitochondrial fission protein1; FITC, fluorescein isothiocyanate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; L-VGCC, L-type voltage-gated calcium channel; Mfn 1/2, mitofusin 1/2; mTOR, mammalian target of Rapamycin; NAC, *N*-acetyl-L-cysteine; NGS, normal goat serum; NMDAR, *N*-methyl-D-aspartate receptor; Opa1, optic atrophy1; PBS, phosphate buffered solution; PKCδ, protein kinase C delta type; ROCK1, Rho-associated coiled-coil-containing protein kinase 1; ROS, reactive oxygen species; RT, room temperature.

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impairs their function [10]. Defects in mitochondria, such as impairment of the electron transport chain (ETC), lead to ATP depletion and ROS generation which are correlated with fragmented mitochondria often found in the brains of AD patients [11,12]. Five key molecules are known to control mitochondrial dynamics: dynamin-related protein 1 (Drp1), mitochondrial fission protein 1 (Fis1), mitofusin 1/2 (Mfn 1/2) and optic atrophy (Opa1) [13,14]. Both the expression levels and post-translational modifications of mitochondrial dynamic proteins influence their protein interactions, localizations, and activations. Post-translational modification by phosphorylation of Drp1 on specific serine residues regulates the activity of Drp1 *via* translocation from cytosol to mitochondria and subsequently resulting in fragmentation of mitochondria [15]. Phosphorylation of serine 616 (S616) and serine 637 (S637) in Drp1 has been extensively studied for their functional role in mitochondrial fission. Drp1 phosphorylation at S616 is mediated by upstream regulators including PKCδ, Erk1/2 and CDK1/5, which promotes mitochondrial fragmentation [16–19]. Another serine residue, S637, phosphorylated by PKA (protein kinase A) prevents mitochondrial fragmentation, while phosphorylation by CaMK1α and ROCK1 exerts functionally opposite effects on Drp1 activity [20–22]. These contradictory effects show that changes in Drp1 activity are controlled by diverse upstream molecules that alter the phosphorylation status of Drp1. Many studies to identify the precise machinery of regulating mitochondrial fragmentation have shown the phosphorylation site of Drp1 regulated by numerous upstream kinases [23,24]. Modification of fission proteins, primarily through phosphorylation, not only induces mitochondrial fission but also affects their function [25]. A recent study investigated how mitochondrial fusion and fission generally occurs [26]. Mitochondrial fission is closely related with mitosis and neuronal excitation [14,27,28]. Linkage of excessive mitochondrial fragmentation and altered signals in AD patients suggest CDK and calcium associated signals as key Drp1 regulators [29,30]. Establishing a fission regulatory pathway could help to overcome the imbalance of mitochondrial dynamics in several neurodegenerative diseases such as AD. However, the precise mechanism of how Aβ-induced mitochondria fragmentation causes the progression of AD remains unclear. This prompted us to find the upstream signal molecules regulating mitochondrial fragmentation.

Several *in vitro* models in human neuroblastoma (NB) cell-lines (SK-N-MC, SK-N-SH, SH-SY5Y) and mouse hippocampal neurons were used in this experiment to examine the mechanism of Aβ-induced mitochondrial alteration. NB cells were used to investigate Aβ-induced mitochondrial dynamics as an in vitro model for AD [31]. The NB cell-line has three different phenotypes: neuroblastic N-type cells, substrate-adherent S-type cells, and intermediate I-type cells [32,33]. Each type of neuronal cell line shows distinctive properties. N-type cells form short neurite processes with small and round morphology. S-type cells are characterized by their large, flat appearance and exhibit strong adherence onto the substrate. I-type cells either have the morphological feature of N- or S-type cell lines, and display moderate adherence and small cell bodies with the presence or absence of neurite processes [34]. We have mainly used I-type of SK-N-MC, which possess both N- and S-type properties. SH-SY5Y and its parental line SK-N-SH were also used to represent N- and S- type NB cells to ensure that the response of Aβ stimulation is not from the different phenotypes of the NB cell-line [35]. Furthermore, because of its similarity to an in vivo model, primary mouse hippocampal neurons were compared with the human cell-line to confirm that they respond in the same manner to Aβ treatment. Aβ-induced Drp1 phosphorylation leading to mitochondrial fission has been implicated in many studies. In the present study, we investigated the signaling pathways that cause both mitochondrial fission and abnormal mitochondrial accumulation that eventually trigger neuronal apoptosis.

2. Materials and methods

2.1. Materials

Human neuroblastoma cell lines were obtained from Korean Cell Line Bank (Seoul, Korea). Fetal bovine serum (FBS) was purchased from Bio Whittaker (Walkersville, MD, USA). Antibodies specific for Mfn 2, Opa1, Drp1, Fis1, caspase-3, caspase-9, p-Akt (Ser 473), Akt, p-CaMKII (Thr 286), CaMKII, CaM, mTOR, Cyt C, Bax, Bcl-2, β-tubulin, and β-actin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-Drp1 (Ser 616), p-mTOR (Ser 2448) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies of LC3 and p62 were obtained from Novus Biologicals (Littleton, CO, USA). COX IV, MAP2 antibodies were obtained from Abcam (Cambridge, England). Mfn 1 antibody was purchased from Proteintech (Wuhan, China). Horseradish peroxidase (HRP)-conjugated IgG was obtained from Jackson Immunoresearch (West Groove, PA, USA). EGTA, Ionomycin, Nifedipine, MK-801, NAC, Rapamycin, Trehalose and 3-MA were purchased from Sigma Chemical Company (St. Louis, MO, USA). Mdivi-1 was purchased from Enzo life sciences (Farmingdale, NY, USA). CB-124005 was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture

Three different human neuroblastoma cell lines were purchased from a Korean cell line bank (Seoul, Korea): SK-N-MC, SK-N-SH and SHSY-5Y were used. Cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Thermo Fisher) supplemented with 1% antibiotics (penicillin/streptomycin)-antimycotics (amphotericin B) and 10% FBS. Cells were grown in 35, 60, or 100 mm diameter culture dishes, or in 12- or 96-well plate at 37 °C with 5% CO_2 in the incubator. After 24 h incubation, cells were cultured at 70–80% confluence, and were transferred to serum-free medium prior to experiments.

2.3. Primary culture of mouse hippocampal neurons

Prenatal mice (18–19 days) were used to isolate hippocampal primary neurons. Isolated hippocampi were minced gently using a sterile scalpel, and treated with trypsin (0.025%). 2.5×10^6 cells were plated at poly-D-lysine coated 35 mm dish in Neurobasal Plating Media (Neurobasal Media containing B27 Supplement [1 ml/50 ml], 0.5 mM Glutamine Solution, 25 μM Glutamate, 0.5% antibiotics-antimycotics, 1 mM HEPES, 10% Heat Inactivated Donor Horse Serum) and placed in an incubator at 37 °C with 5% CO_2. After 24 h, growth media was changed to Neurobasal Feeding Media (Neurobasal Media containing B27 Supplement [1 ml/50 ml], 0.5 mM Glutamine Solution, 0.5% antibiotics, 1 mM HEPES. Cytosine arabinoside (AraC) was treated to avoid glial cell early time points in the culture [36]. MAP2 was purchased from Abcam (Cambridge, England) and used for staining neuronal cytoskeletal protein (Supplementary Fig. 1A).

2.4. Aβ oligomerization and treatment

The β-Amyloid [1–42] (Human) peptide was purchased from Invitrogen Corporation (Camarillo, CA, USA). 1 mg of the peptide was dissolved in 1 ml of a 100% HFIP (1,1,1,3,3,3-hexafluoro-2-propanol [Sigma Chemical Company (St. Louis, MO, USA)]) and incubated for 1 h at room temperature with occasional vortexing. This was followed by sonication for 10 min in a water bath and then freeze-drying for 3 h. After film formation, the peptide was dissolved in 100% DMSO (1 mM). PBS (100 μM) was added for dilution, and then incubated for 24 h at 4 °C to oligomerize Aβ as described previously [37]. After 24 h of serum starvation, Aβ oligomers were added at a final concentration of 5 μM in the media.