Biochimica et Biophysica Acta 1842 (2014) 220–231

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Inhibition of ERK-DLP1 signaling and mitochondrial division alleviates mitochondrial dysfunction in Alzheimer's disease cybrid cell

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ARTICLE INFO

Article history:
Received 1 July 2013
Received in revised form 27 October 2013
Accepted 11 November 2013
Available online 16 November 2013

Keywords:
Mitochondrial fission and fusion
Alzheimer's disease
Cybrid cells
ERK
DLP1

ABSTRACT

Mitochondrial dysfunction is an early pathological feature of Alzheimer's disease (AD). The underlying mechanisms and strategies to repair it remain unclear. Here, we demonstrate for the first time the direct consequences and potential mechanisms of mitochondrial functional defects associated with abnormal mitochondrial dynamics in AD. Using cytoplasmic hybrid (cybrid) neurons with incorporated platelet mitochondria from AD and age-matched non-AD human subjects into mitochondrial DNA (mtDNA)-depleted neuronal cells, we observed that AD cybrid cells had significant changes in morphology and function; such changes associate with altered expression and distribution of dynamin-like protein (DLP1) and mitofusin 2 (Mfn2). Treatment with antioxidant protects against AD mitochondria-induced extracellular signal-regulated kinase (ERK) activation and mitochondrial fission-fusion imbalances. Notably, inhibition of ERK activation not only attenuates aberrant mitochondrial morphology and function but also restores the mitochondrial fission and fusion balance. These effects suggest a role of oxidative stress-mediated ERK signal transduction in modulation of mitochondrial fission and fusion events. Further, blockade of the mitochondrial fission protein DLP1 by a genetic manipulation with a dominant negative DLP1 ($DLP1^{K38A}$), its expression with siRNA-DLP1, or inhibition of mitochondrial division with mdivi-1 attenuates mitochondrial functional defects observed in AD cybrid cells. Our results provide new insights into mitochondrial dysfunction resulting from changes in the ERK-fission/fusion (DLP1) machinery and signaling pathway. The protective effect of mdivi-1 and inhibition of ERK signaling on maintenance of normal mitochondrial structure and function holds promise as a potential novel therapeutic strategy for AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia characterized clinically progressive cognitive decline and neuronal loss. Pathologically, AD-affected brain shows accumulation of amyloid beta peptide (Aβ) and neurofibrillary tangles with tau hyperphosphorylation. Recent studies indicate that mitochondrial dysfunction, an early pathological feature in AD, plays a central role in its pathogenesis of AD [1–6]. Perturbed bioenergetic function, especially mitochondrial dysfunction, is seen in brain and peripheral tissues of subjects with AD [7,8]. Cytochrome c oxidase (CcO) activity is lower in human AD platelet mitochondria [8–10]. Neurons are especially vulnerable to mitochondrial dysfunction due to inherent high energy demands and dependence on respiration for ATP generation [3,11]. Thus, mitochondrial dysfunction may drive or mediate various AD pathologies.

Mitochondria are dynamic organelles that undergo continuous fission and fusion. These processes are regulated by the large dynamin-related GTPases mitofusin 1 and 2 (Mfn1 and 2), and optic atrophy1 (OPA1) for fusion and dynamin-like protein (DLP1) for fission [12,13]. Mitochondrial dynamics play an essential role in ensuring appropriate distribution of mitochondria within cells, a function that is particularly critical for morphologically complex cells such as neurons [14]. Alterations in mitochondrial dynamics significantly impact almost all aspects of mitochondrial function including energy metabolism, calcium buffering, reactive oxygen species (ROS) generation and apoptosis regulation [12,15]. Imbalance of mitochondrial fission and fusion is an important mechanism in neurodegenerative diseases including Parkinson disease (PD), Huntington disease (HD), and AD. Although it has been demonstrated that altered mitochondrial fission or fusion is involved in Aβ-mediated mitochondrial morphological

Abbreviations: AD, Alzheimer's disease; mtDNA, Mitochondrial DNA; DLP1, Dynamin-like protein; Mfn2, Mitofusin 2; ERK, Extracellular signal-regulated kinase; Aβ, Amyloid beta peptide; CcO, Cytochrome *c* oxidase; ROS, Reactive oxygen species; PD, Parkinson disease; HD, Huntington disease; TMRM, Tetramethylrhodaminemethylester

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http://dx.doi.org/10.1016/j.bbadis.2013.11.009

changes leading to neuronal and synaptic dysfunction in a transgenic AD mouse model and *in vitro* cell culture [15–17], the direct consequences and mechanisms underlying AD-derived mitochondrial defects on mitochondrial dynamics and associated mitochondrial function have not been fully elucidated. The following questions arise: Do AD-derived mitochondria show changes in mitochondrial fission and fusion events? If so, are these altered mitochondrial dynamics associated with mitochondrial dysfunction? Does inhibition of abnormal mitochondrial fusion and fission rescue aberrant mitochondrial morphology and function? Thus, it is essential to uncover the mechanism by which AD mitochondria modulate this vital mitochondrial process.

To explore the mechanisms associated with AD-specific mitochondrial defects, we used cybrid cells with incorporated platelet mitochondria from AD or age-matched non-AD human subjects into mitochondrial DNA (mtDNA)-depleted neuronal cells (SH-SY5Y). The resulting cell lines, referred to as AD or non-AD cybrids, have been demonstrated to have different bioenergetic profiles [7]. AD cybrids recapitulate many potential pathogenic features of AD, such as decreased activity associated with respiratory chain key enzyme, increased free radical production rates, and other functional changes that likely arise as a consequence of perturbed respiratory chain function typically observed in AD brain mitochondria [18,19].

Using AD cybrids, we comprehensively evaluated the consequences of changes in AD-specific mitochondria on mitochondrial dynamics and mitochondrial function. We further delineated the mechanism by which AD mitochondria regulate mitochondrial fission/fusion events. Our investigation provides new insight into the role of mitochondrial dynamics in AD pathogenesis, highlighting the potential diagnostic and therapeutic application for AD.

2. Materials and methods

2.1. Human subjects and creation of cybrid cell lines

Individuals for this study were recruited from the University of Kansas Alzheimer's Disease Center. AD subjects met the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria [20]. Non-AD subjects were cognitively normal and age-matched to AD subjects. This study was approved by the University of Kansas Medical Center (KUMC) Institutional Review Board. All subjects provided written informed consent to participate in the study. The ages of AD and non-AD subject platelet donors were 73.3 ± 2.6 and 74 ± 2.9 years, respectively. Gender, age and disease status of donor patients are presented in supplemental Table. S1.

Cybrid cell lines were created on the human neuroblastoma cell (SH-SY5Y) nuclear background (by the KU ADC Mitochondrial Genomics and Metabolism Core) [21]. To create the cybrid cell lines used for this study, SH-SY5Y cells that were previously depleted of endogenous mtDNA (Rho0 cells) were fused with the platelet cytoplasm and repopulated with mitochondria containing mtDNA from patients or controls as previously described [22]. Briefly, Rho0 cells were incubated with donor platelets in a DMEM-polyethylene glycol solution. Immediately after this, cells were initially placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% non-dialyzed fetal bovine serum (FBS), 200 μg/ml sodium pyruvate, 150 μg/ml uridine, and 1% penicillin–streptomycin solution to recover. Seven days after the fusion event, cells were switched to a selection medium containing 10% dialyzed fetal calf serum but lacking pyruvate and uridine. These conditions resulted in selection against Rho0 cells that were not repopulated with donor mitochondria. Only cells containing patient's platelet mtDNA can regain aerobic competence and survive the subsequent selection processes. Following selection, each cybrid cell line was maintained in medium containing DMEM supplemented with 10% non-dialyzed FBS and 1% penicillin-streptomycin solution in a humidified 95% air/5% CO_2 incubator at 37 °C for over 2 months. The quantitative real-time PCR showed that the intact mtDNA copies were present in all cybrids without detectable large scale deletion after many passages of cell proliferation (Fig. S1).

Cells were treated with probucol (10 μM) (Sigma), ERK1/2 inhibitor PD98058 (10 μM) (Sigma), or mitochondrial division inhibitor mdivi-1 (10 μM) (Sigma) for 24 h prior to biochemical and molecular assays.

2.2. Measurement of enzyme activities associated with respiratory chain complexes

Briefly, cybrid cells were washed with ice-cold PBS, and then harvested, centrifuged, and suspended in 50 μL of isolation buffer containing 250 mM sucrose, 20 mM HEPES, and 1 mM EDTA. Cell suspensions (containing ~3–4 mg of protein/ml) were added to a cuvette containing 0.95 mL of 1× assay buffer (10 mM Tris–HCl, and 120 mM KCl), and the reaction volume was brought to 1.05 mL with the addition of 1× enzyme dilution buffer (10 mM Tris–HCl, pH 7.0). The reaction was then initiated by addition of 50 μL of ferrocytochrome substrate solution (0.22 mM), and the change in absorbance of cytochrome c at 550 nm was measured using a Shimadzu (Kyoto, Japan) UV1200 spectrophotometer. Activity is expressed as micromoles of cytochrome oxidized per min^{-1} mg^{-1} protein using an extinction coefficient of 27.84 mM^{-1} cm^{-1}. Enzyme activities in complex I (NADH-ubiquinone reductase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase, CcO) and citrate synthase activity were determined as described previously [23,24].

2.3. Measurement of ATP levels

ATP levels were determined using an ATP Bioluminescence Assay Kit (Roche) following the manufacturer's instructions [2,25]. Briefly, cells were harvested using the provided lysis buffer, incubated on ice for 15 min, and centrifuged at 13,000*g* for 10 min. ATP levels were measured using a Luminescence plate reader (Molecular Devices) with an integration time of 10 s.

2.4. Functional imaging

Cybrid cells were harvested from 75 cm^2 flasks and replated at low density onto Lab-Tek eight-well chamber slides. Mitochondrial ROS generation was determined using Mitosox Red (Molecular Probes), a unique fluorogenic dye highly selective for detection of superoxide production in live cell mitochondria. Cells were incubated with fresh growth medium containing 2.5 μM Mitosox for 30 min. For mitochondrial membrane potential determination, cells were co-stained with Mitotracker Green (MTGreen) (100 nM; Molecular Probes) and TMRM (100 nM; Molecular Probes) for 30 min. Fluorescence from MTGreen is independent of membrane potential, whereas TMRM is sensitive to membrane potential. Mitochondria were labeled with Mitotracker Red (Molecular Probes, incubated in 100 nM Mitotracker Red for 30 min at 37 °C before fixation) to visualize morphology.

Images were captured under a microscope (Leica TCS SPE) using a 63X 1.4 NA Apochrome objective (Carl Zeiss MicroImaging, Inc.). Excitation wavelengths were 543 nm for Mitosox, TMRM or Mitotracker Red, and 488 nm for MTGreen, respectively. Fluorescent signals were quantified using NIH Image J software. Post-acquisition processing was performed with MetaMorph (Molecular Devices) and NIH Image J software for quantification and measurement of fluorescent signals of mitochondrial length and occupied area. Mitochondrial size, shape, density, and fluorescent intensity were quantified by an investigator blinded to experimental groups. More than 100 clearly identifiable mitochondria from randomly selected 10–15 cells per experiment were measured in 3 independent experiments.