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Original Contribution

Oxidative stress-mediated activation of extracellular signal-regulated kinase contributes to mild cognitive impairment-related mitochondrial dysfunction

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ABSTRACT

Mild cognitive impairment (MCI) occurs during the predementia stage of Alzheimer disease (AD) and is characterized by a decline in cognitive abilities that frequently represents a transition between normal cognition and AD dementia. Its pathogenesis is not well understood. Here, we demonstrate the direct consequences and potential mechanisms of oxidative stress and mitochondrial dynamic and functional defects in MCI-derived mitochondria. Using a cytoplasmic hybrid (cybrid) cell model in which mitochondria from MCI or age-matched non-MCI subjects were incorporated into a human neuronal cell line depleted of endogenous mitochondrial DNA, we evaluated the mitochondrial dynamics and functions, as well as the role of oxidative stress in the resultant cybrid lines. We demonstrated that increased expression levels of mitofusin 2 (Mfn2) are markedly induced by oxidative stress in MCI-derived mitochondria along with aberrant mitochondrial functions. Inhibition of oxidative stress rescues MCI-impaired mitochondrial fusion/fission balance as shown by the suppression of Mfn2 expression, attenuation of abnormal mitochondrial morphology and distribution, and improvement in mitochondrial function. Furthermore, blockade of MCI-related stress-mediated activation of extracellular signal-regulated kinase (ERK) signaling not only attenuates aberrant mitochondrial morphology and function but also restores mitochondrial fission and fusion balance, in particular inhibition of overexpressed Mfn2. Our results provide new insights into the role of the oxidative stress–ERK–Mfn2 signal axis in MCI-related mitochondrial abnormalities, indicating that the MCI phase may be targetable for the development of new therapeutic approaches that improve mitochondrial function in age-related neurodegeneration.

Mild cognitive impairment (MCI) is characterized by a decline in cognitive abilities that is noticeable, yet not severe enough to completely disrupt an individual's daily activity, and is generally considered to be a transitional phase between normal aging and early dementing disorders, especially Alzheimer disease (AD) [1–3]. As patients with MCI typically convert to definitive AD (probability of 50% within 4 years or at a rate of about 12% per year) [4,5], it is important to consider whether targeting early treatment interventions toward MCI patients holds potential merit. Some neuropathologic changes observed in MCI partially overlap with the functional alterations seen in AD; for example, neuritic plaques in neocortical regions and neurofibrillary tangles in the temporal lobe are seen in MCI patients [6], and there is significant elevation of oxidative DNA damage in peripheral lymphocytes and brain tissues of MCI patients [7–9]. In addition, several gene mutations associated with AD have been observed in subjects with MCI, including polymorphic variation in apolipoprotein E and mutations in presenilin 1 and the amyloid precursor protein [10–13]. Although the underlying mechanisms remain elusive, increasing evidence indicates an essential role for mitochondrial dysfunction in AD etiology and pathology [14]. Interestingly, the impairment of mitochondrial function observed in AD is also seen in MCI subjects; this includes decreased cytochrome *c* oxidase activity [15], decreased mitochondrial membrane potential, and lower

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mitochondrial cytochrome *c* content [16]. Thus, although we know that mitochondrial dysfunction may play a critical role in MCI pathologies and its development into AD, its underlying mechanisms are not well understood.

Mitochondria are dynamic organelles, which engage in repeated cycles of fusion and fission. Mitochondrial dynamics (fission and fusion events) are essential for maintenance of mitochondrial morphology, appropriate distribution, and normal function [17,18]. In mammals, the balance of mitochondrial dynamics is regulated by the large dynamin-related GTPase fusion (mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (Opa1)) and fission (dynamin-like protein (Drp1) and mitochondrial fission 1 protein (Fis1)) proteins [19,20]. Neurons are particularly reliant on mitochondrial dynamic properties as they require mitochondria in the synaptic terminals [21]. Deficiency in either fission or fusion reduces mitochondrial trafficking, leading to aberrant distribution of mitochondria and defective cellular function [22,23]. Disrupted mitochondrial fission/fusion balance is consistently involved in neurodegenerative diseases, including AD [24,25]. Although altered balance of mitochondrial fission/fusion is involved in AD postmortem brain [25,26], transgenic AD mouse models, and amyloid β-treated in vitro cell cultures [27,28], the role of the mitochondrial dynamic balance in mediating MCI mitochondrial morphology and function and its underlying mechanisms have not been explored.

In this study, we determined whether and how mitochondrial alterations occur in MCI-derived mitochondria. Using the cytoplasmic hybrid (cybrid) model in which mitochondria from MCI patients or symptom-free, age-matched non-MCI subjects were incorporated into human neuronal (SH-SY5Y) cells previously depleted of endogenous mitochondrial DNA (mtDNA), we comprehensively evaluated changes in MCI-specific mitochondrial dynamics and mitochondrial functions. Our studies provide substantial evidence that disturbed mitochondrial dynamics and impaired mitochondrial functions contribute to MCI pathology and may provide an opportunity for developing diagnostic and therapeutic advances.

Material and methods

Human subjects and creation of cybrid cell lines

Human subjects for the MCI and non-MCI group were recruited from the University of Kansas Alzheimer's Disease Center (KU ADC; seven MCI patients and seven age-matched non-MCI controls). Based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria [29], MCI diagnosis was made in accordance with the criteria defined by Petersen et al. [5], and the patients were classified as 0.5 according to the Clinical Dementia Rating scale. Non-MCI subjects were without subjective or objective evidence of cognitive impairment. The ages of MCI and non-MCI subject platelet donors were 72.6 ± 2.5 and 74 ± 3.0 years, respectively. Gender, age, and disease status of donor patients are presented in Supplementary Table S1. This study was approved by the University of Kansas Medical Center Institutional Review Board. All subjects provided written informed consent to participate in the study.

To create cybrids for this study, Rho^0 SH-SY5Y cells lacking mtDNA were obtained from the KU ADC Mitochondrial Genomics and Metabolism Core and repopulated with mitochondria containing platelet mtDNA from volunteer patients or age-matched controls as previously described [30]. Briefly, Rho^0 cells were incubated with donor platelets in a Dulbecco's modified Eagle medium (DMEM)–polyethylene glycol solution. Immediately after this, cells were initially placed in DMEM supplemented with 10% nondialyzed 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, the cells were switched to a selection medium containing 10% dialyzed fetal calf serum without pyruvate and uridine. These conditions resulted in selection against Rho^0 cells that were not repopulated with donor mitochondria. Only cells containing patient platelet mtDNA can regain aerobic competence and survive the subsequent selection processes. After selection, each cybrid cell line was maintained in DMEM supplemented with 10% nondialyzed FBS and 1% penicillin–streptomycin solution in a humidified 95% air, 5% CO_2 incubator at 37 °C for over 2 months. Quantitative real-time polymerase chain reaction showed that intact mtDNA copies were present in all cybrids without detectable large-scale deletion after many passages of cell proliferation (Supplementary Fig. S1). Cells were treated with the antioxidant drug probucol (10 μM; Sigma) and the extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98058 (10 μM; Sigma) for 24 hs before biochemical and molecular assays.

Measurement of enzyme activities associated with respiratory chain complexes and ATP levels

The key enzyme activities associated with the mitochondrial respiratory chain, including complex I (NADH-ubiquinone reductase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome *c* reductase), complex IV (cytochrome *c* oxidase, CcO), and citrate synthase, were measured in cybrid cell lysates and isolated platelets' mitochondria as described previously [31–33]. ATP levels were measured using an ATP Bioluminescence Assay Kit (Roche) following the manufacturer's instructions and using a Shimadzu (Kyoto, Japan) UV1200 spectrophotometer.

Oxidative stress, mitochondrial membrane potential, and mitochondrial morphology analysis

Cybrid cells were harvested from 75-cm^2 flasks and replated at low density onto Lab-Tek eight-well chamber slides. To estimate production of reactive oxygen species (ROS), 80% confluent cybrid cells were exposed to 2.5 μM MitoSOX red, a fluorochrome specific for anion superoxide produced in the inner mitochondrial compartment (Molecular Probes), at 37 °C for 30 min. To assess mitochondrial membrane potential, cells were costained with MitoTracker green (MTGreen; 100 nM; Molecular Probes) and tetramethylrhodamine methyl ester (TMRM; 100 nM; Molecular Probes) at 37 °C for 30 min. Fluorescence from MTGreen is independent of the membrane potential, whereas TMRM is sensitive to the membrane potential. For visualization of mitochondria, cybrid cells were stained with MitoTracker red (200 nM; Molecular Probes) at 37 °C for 30 min before fixation.

Leica TCS SPE confocal scanning microscopes with a 63 × 1.4 NA Apochrome objective lens (Carl Zeiss MicroImaging, Inc.) were used to photograph cells. Excitation wavelengths were 543 nm for MitoSOX, TMRM, or MitoTracker red, and 488 nm for MTGreen. Postacquisition processing was performed with MetaMorph (Molecular Devices) and NIH Image J software for quantification and measurement of fluorescent signals to assess mitochondrial length and occupied area. Mitochondrial size, shape, density, and fluorescence intensity were quantified by an investigator blinded to experimental groups. More than 100 clearly identifiable mitochondria from 10 to 15 randomly selected cells per experiment were measured.

Evaluation of intracellular ROS levels was accessed by election paramagnetic resonance (EPR) spectroscopy as described in our previous study [34–36]. Cyclic hydroxylamine 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (100 μM) was added to the cybrid cell culture 30 min before the end of the treatments.