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Research Paper

Mitochondrial permeability transition pore contributes to mitochondrial dysfunction in fibroblasts of patients with sporadic Alzheimer's disease

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ABSTRACT

In the last few decades, many reports have suggested that mitochondrial function impairment is a hallmark of Alzheimer's disease (AD). Although AD is a neurodegenerative disorder, mitochondrial damage is also present in patients' peripheral tissues, suggesting a target to develop new biomarkers. Our previous findings indicate that AD fibroblasts show specific defects in mitochondrial dynamics and bioenergetics, which affects the generation of adenosine triphosphate (ATP). Therefore, we explored the possible mechanisms involved in this mitochondrial failure. We found that compared with normal fibroblasts, AD fibroblasts had mitochondrial calcium dysregulation. Further, AD fibroblasts showed a persistent activation of the non-specific mitochondrial calcium channel, the mitochondrial permeability transition pore (mPTP). Moreover, the pharmacological blockage of mPTP with Cyclosporine A (CsA) prevented the increase of mitochondrial superoxide levels, and significantly improved mitochondrial and cytosolic calcium dysregulation in AD fibroblasts. Finally, despite the failure of CsA to improve ATP levels, the inhibition of mitochondrial calcium uptake by the mitochondrial calcium uniporter increased ATP production in AD fibroblasts, indicating that these two mechanisms may contribute to mitochondrial failure in AD fibroblasts. These findings suggest that peripheral cells present similar signs of mitochondrial dysfunction observed in the brain of AD patients. Therefore, our work creates possibilities of new targets to study for early diagnosis of the AD.

1. Introduction

AD is typically a late-onset disorder and represents the most common form of dementia among the aging population [17]. AD is characterized by the presence of different aggregates of misfolded proteins in the brain, namely amyloid beta peptides (Aß) and neurofibrillary tangles (NFTs); these tangles are generated by pathological forms of the tau protein [30]. Another important hallmark of AD is mitochondrial dysfunction, which regularly appears prior to tau and Aß pathology [30]; this mitochondrial dysfunction contributes to the synaptic and neuronal damage observed in AD [37,5].

One of the principal functions of the mitochondria is to convert energy derived from nutrients into adenosine triphosphate (ATP) [23]. Current evidence suggests that in AD brains, mitochondrial dysfunction is commonly presented as a reduction in ATP synthesis, increase in reactive oxygen species (ROS) production, impairment of calcium homeostasis, and an imbalance in mitochondrial dynamics [22,5]. More importantly, mitochondrial damage is not only an early and progressive feature present in the AD brain, but also has been found in peripheral tissues derived from AD patients [27,34,37,7]. Our prior research suggests that fibroblasts obtained from AD patients demonstrate a significant alteration in mitochondrial length and dynamics [27]. Additionally, AD fibroblasts show an impaired mitochondrial bioenergetics profile compared to aged-matched and young patients' cells [27].

Abbreviations: AD, Alzheimer's disease; ADP, Alzheimer's disease patient; CDR, Clinical Dementia Rating; DCF, chloromethyl-2,7-dichlorodihydrofluorescein diacetate; ER, endoplasmic reticulum; NP, normal patient; MAM, mitochondrial associated membranes; MMP, mitochondrial membrane potential; MoCA, Montreal Cognitive Assessment; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; TMRM, tetramethyl-rhodamine methyl ester

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Despite our findings suggesting that peripheral fibroblasts could replicate the mitochondrial failure observed in the AD brain, the underlying mechanisms are currently unknown.

Recent studies have suggested that the mitochondrial permeability transition pore (mPTP) could be playing a role in the metabolic stress observed in AD [10,16,28]. The genetic removal of the Cyclophilin D (CypD) protein, a key component of the mPTP, prevents against mitochondrial dysfunction and improves synaptic and cognitive loss. This is seen in transgenic AD models that demonstrate the overexpression of an amyloid precursor protein (APP) [10,9]. Furthermore, cultured neurons obtained from CypD knock-out mice showed a decrease in Aβ-dependent ROS generation, increase in the calcium buffering capacity, improved mitochondrial respiratory function, and attenuation of abnormalities in synaptic plasticity present in AD [10,16]. These findings suggest opening of the mPTP could be responsible for the mitochondrial dysfunction observed in the AD brain; however, its contribution to mitochondrial failure in AD peripheral cells remains elusive. Therefore, we studied the contribution of the mPTP and calcium dysregulation in mitochondrial impairment present in fibroblasts obtained from AD and aged-matched patients.

2. Methods

2.1. Patients and cell culture fibroblasts

Skin fibroblasts were obtained from six AD-patients and five age-matched healthy controls, and they were cultured in growth media containing MEMα (Biological Industries), with 10% FBS (Gibco) and 1% penicillin-streptomycin (Corning) [35]. To prevent mPTP opening, cells were pre-treated with 0.5 μM Cyclosporine A (CsA; Tocris, Bioscience) for 2 h, as recommended by the manufacturer. To study mitochondrial calcium uniporter (MCU) participation, cells were treated with 20 μM Ruthenium Red (RRed; Tocris, Bioscience) for 1 h, as indicated. All patients were recruited after providing informed consent, and the study was approved by the Ethics Committee of the Hospital Clínico de la Universidad de Chile and Universidad Autónoma de Chile. AD diagnosis was established according to tests by the National Institute of Neurological and Communicative Diseases and Stroke-AD and Related Disorders Association [24], as well as the Clinical Dementia Rating (CDR) scale, as shown in Table 1 [25]. For the age of the patients, fibroblasts donors are not believed to carry any autosomal dominant mutations as the main cause of AD. The APOE genotype presence in AD and age-match non-demented fibroblasts is unknown.

This table represents the number of patients, age range, sex, Montreal Cognitive Assessment (MoCA) test scores, and diagnosis type for each patient according to the cognitive tests applied. The maximum score for the MoCA is 30, with lower scores associated with greater cognitive deterioration.

2.2. Determination of mitochondrial superoxide and ROS levels

ROS levels were evaluated using chloromethyl-2,7-dichlorodihydrofluorescein diacetate (DCF) dye (Molecular Probes, OR, USA); mitochondrial superoxide levels were determined using MitoSOX Red (Molecular Probes, OR, USA) in conjunction with the mitochondrial marker Mitotracker Green (MGreen; Molecular Probes, OR, USA). Cultured fibroblasts were incubated with 10 μM DCF, 0.5 μM MitoSOX, or 100 nM MGreen in Krebs-Ringer-HEPES (KRH)-glucose buffer at 37 °C for 30 min [32]. Images were taken by adjusting to the same exposure time and gain detector to diminish the photo-bleaching effect of the dye [32]. Quantification of fluorescence intensity for each separate experiment was carried out by analyzing the signal intensity of 25 images for every indicated condition, using Image J software. Results from the three independent experiments were expressed as the average fluorescence intensity, per area, in every image. Fluorescence images were taken with a high-resolution fluorescence microscopy (Leica LX6000, Germany) using a 63x oil objective.

2.3. Mitochondrial and cytosolic calcium measurements

Cytosolic calcium levels were evaluated using Fluo-3AM dye (Molecular Probes, OR, USA), and mitochondrial calcium levels were determined using Rhod-2AM (Molecular Probes, OR, USA) in conjunction with MGreen. Fibroblasts were loaded with 1 μM Fluo-3 AM, or 0.5 μM Rhod-2AM with 0.1 μM MGreen in KRH-glucose buffer at 37 °C for 30 min [32]. For basal mitochondrial calcium measurements, Rhod-2 fluorescence intensity was determined from at least 25 images for every indicated condition, using Image J software. Results from the three independent experiments were expressed as the average fluorescence intensity, per area, in every image. To determine changes in calcium levels in basal conditions, we analyzed the fluorescence intensity (Fluo-3 or Rhod-2) of 5 images in each experiment for every independent condition. To determinate dynamic calcium changes, we treated the cells with thapsigargin (10 μM) for 20 min and we measure fluorescence intensity (Fluo-3 or Rhod-2) from at least 15 cells on average, per experiment. Fluorescent background was subtracted from the dye fluorescence measurements in every experiment, as previously described [32]. Fluorescence intensity and quantification of the three independent experiments were made using Image J software (NIH).

2.4. Determination of ATP levels

Total ATP levels were measured in fibroblasts whole lysate using a luciferin/luciferase bioluminescence assay kit (ATP Determination Kit #A22066, Molecular Probes, Invitrogen). The amount of ATP in each sample was calculated from standard curves and normalized to the total protein concentration.

2.5. Reverse transcription and real-time PCR

RNA was isolated from cells using TRIZOL (Invitrogen, Life Technologies) and eluted in RNase free water, according to the manufacturer's protocol. Extracted RNA was treated with RNase free-DNase I, Amplification Grade, (Invitrogen) to remove traces of contaminating DNA and it was then heat-treated to inactivate DNase I and precipitated with ethanol to clean up the reaction. One microgram of RNA was subjected to reverse transcription using ImProm-II Reverse Transcription System (Promega), following the manufacturer's protocol.

A real-time polymerase chain reaction (PCR) was performed in triplicate in the LightCycler® 96 System (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using KAPA SYBR FAST qPCR Master Mix (2 ×). Amplification conditions consisted of an initial hot start at 95 °C for 10 min, followed by amplification of 40 cycles (95 °C

Table 1
Demographic characteristics of the patients.

Abbreviations	Number of patients	Range Age	Percent women	MoCA Test range	Diagnostic
NP	**5**	**70–85**	**100**	**25–30**	Control
MCI	**2**	**75–85**	**100**	**13–19**	Mild cognitive decline AD
ADP	**4**	**65–80**	**50**	**0–6**	Severe cognitive decline AD