Mitochondrion 13 (2013) 298–311

Contents lists available at SciVerse ScienceDirect

Mitochondrion

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

Mitochondrial respiratory chain Complexes I and IV are impaired by β-amyloid *via* direct interaction and through Complex I-dependent ROS production, respectively

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ARTICLE INFO

Article history:
Received 30 October 2012
received in revised form 5 February 2013
accepted 26 March 2013
Available online 4 April 2013

Keywords:
β-Amyloid
Mitochondria
Respiratory chain complexes
ROS
Cerebellar granule cells

ABSTRACT

Here we investigate the effect of β-amyloid on mitochondrial respiratory function, *i.e.* mitochondrial oxygen consumption and membrane potential generation as well as the individual activities of both the mitochondrial Complexes I–IV, that compose mitochondrial electron transport chain, and the ATP synthase, by using homogenate from cerebellar granule cells, treated with low concentrations of β-amyloid, and Alzheimer synaptic-enriched brain samples. We found that β-amyloid caused both a selective defect in Complex I activity associated with an increase (5 fold) of intracellular reactive oxygen species and an impairment of Complex IV likely due to membrane lipid peroxidation. In addition, a 130% increase of the GSSG/GSH ratio was measured in Alzheimer brains with respect to age-matched controls. Knowing the mechanisms of action of β-amyloid could allow to mitigate or even to interrupt the toxic cascade that leads a cell to death. The results of this study represent an important innovation because they offer the possibility to act at mitochondrial level and on specific sites to protect cells, for example by preventing the interaction of β-amyloid with the identified targets, by stabilizing or by restoring mitochondrial function or by interfering with the energy metabolism.

1. Introduction

Along with synapses loss, the mitochondrial dysfunction not only is an early feature of Alzheimer's disease (AD) (Lin and Beal, 2006), but defective energy metabolism has also a causative role in AD (Manczak et al., 2004, 2006; Mosconi et al., 2008; Valla et al., 2001). Notably, recent studies have highlighted a pivotal role of mitochondrial β-amyloid (Aβ) in AD synaptic damage and in cognitive decline (for references see Chen and Yan, 2010; Eckert et al., 2011; Reddy et al., 2010): Aβ is able to pass through both the outer cell membrane and the mitochondrial membrane (for reference see Chen and Yan, 2010), to interact with several proteins inside mitochondria and to lead to abnormalities of mitochondrial function (for references see Gouras et al., 2010), such as decreased activity of respiratory chain (RC) and citric acid cycle enzymes and generation of reactive oxygen species (ROS) (Casley et al., 2002; Caspersen et al., 2005; Chen and Yan, 2010; Manczak et al., 2004). Furthermore, Aβ is associated with mitochondria from AD patients, compared to a cohort subjects (Du et al., 2008; Lustbader et al., 2004), and mitochondria-specific accumulation of Aβ is sufficient to induce mitochondria impairment and neuronal death as that observed in AD mice or in Aβ-treated human neurons (Cha et al., 2012). In particular, proteomic studies using triple transgenic mice (pR5/APP/PS2) showed a massive deregulation of several proteins, one third of which were mitochondrial proteins mainly related to Complexes I and IV of the oxidative phosphorylation (OXPHOS) system (Rhein et al., 2009). Similar findings have also been reported in post-mortem brain tissues, as well as in other tissues, such as platelets from AD patients and AD cybrid cells (Cardoso et al., 2004a,b), but to date no proposals have been made to explain how Aβ impairs the complexes.

Recently we have proved that Aβ peptide(s) interact(s) with a neurotoxic NH_2-derived tau fragment of the human tau40 isoform (441 amino acids) in human AD synapses in association with mitochondrial adenine nucleotide translocator (ANT-1). The 2 peptides — *i.e.* Aβ1–42 and the smaller tau peptide NH_2-26-44 — inhibit the ANT-1 in a non-competitive and competitive manner, respectively, and together further aggravate the mitochondrial dysfunction by exacerbating the ANT-1 impairment, thus leading to dysfunction in energy metabolism prior to induction of cell death (Amadoro et al., 2006, 2012; Atlante et al., 2008).

Abbreviations: AA, antimycin A; Aβ, β-amyloid; AD, Alzheimer disease; ASC, ascorbate; CGCs, cerebellar granule cells; Co-IP, co-immunoprecipitation; COX, cytochrome oxidase; Cyt *c*, cytochrome *c*; DCIP, dichloroindophenol; DQH_2, duroquinol reduced; DQox, duroquinone oxidized; ETC, electron transport chain; GSH, glutathione; GSSG, glutathione disulphide; KCN, potassium cyanide; MALO, malonate; OLIGO, oligomycin; OXPHOS, oxidative phosphorylation; PBS, phosphate buffer saline medium; PEP, phosphoenol pyruvate; PK, pyruvate kinase; PnAc, *cis*-parinaric acid; RC, respiratory chain; ROS, reactive oxygen species; S.D., standard deviation; SUCC, succinate; TMPD, tetramethyl p-phenylenediamine; XX, xanthine; XOD, xanthine oxidase.

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1567-7249/$ – see front matter
http://dx.doi.org/10.1016/j.mito.2013.03.008

In order to unravel the direct impact of Aβ on mitochondrial respiratory function, mitochondrial oxygen consumption as well as the individual activities of both the mitochondrial Complexes I–IV and the ATP synthase, *i.e.* Complex V, were here analyzed by using homogenate from cerebellar granule cells (CGCs), treated with low concentrations of Aβ in the micromolar range 0.5–2 μM (for reference see Amadoro et al., 2012), and AD synaptic-enriched brain samples. We found that Aβ caused a selective defect in Complex I activity associated with an increase of ROS and an impairment of Complex IV likely due to the deleterious action of ROS. The obtained results increase the knowledge of the mechanisms of mitochondrial dysfunction related to the pathogenesis of AD and provide further information for developing the mitochondria-targeted therapeutic strategy for AD.

2. Materials and methods

2.1. Ethics statements

This study was performed in accordance with local ethics committee and with the principles contained in the Declaration of Helsinki as revised in 1996. All animals were handled and cared for in accordance with EEC guidelines (Directive 86/609/CEE).

2.2. Reagents

Tissue culture medium and fetal calf serum were purchased from Gibco (Grand Island, NY, USA) and tissue culture dishes were from NUNC (Taastrup, Denmark). All enzymes and biochemicals, as well cytochrome *c* oxidase from bovine heart, were from Sigma Chemical Co. (St Louis, MO, USA). Antibody against NDUFS3 (17D95) (c-58393) is from Santa Cruz Biotecnology and anti-Aβ protein/APP 4G8 (aa 17–24) mouse MAB1561 from Chemicon Temecula (CA).

Fibrillar Aβ1–42 (Sigma Chemicals Co., St. Louis, MO, USA) — sometimes called, for simplicity, Aβ — was prepared according to Eckert et al. (2008) with minor modifications. The peptide was dissolved in deionized water at concentration of 0.5 mM and stored at −20 °C. At occurrence, the stock solution was diluted in phosphate-buffered saline (PBS) to a concentration of 0.1 mM and incubated at 37 °C, with gentle agitation, for 24 h to obtain aged, aggregated preparations of Aβ1–42.

2.3. Brain material

Human brain material was provided *via* the rapid autopsy program of the Netherlands Brain Bank (NBB), which provides postmortem specimens from clinically well documented and neuropathologically confirmed cases. All research involving them was conducted according to the ethical declaration of the NBB. All cases were neuropathologically confirmed, using conventional biochemical and histopathological techniques (see Amadoro et al., 2012), and diagnosis performed using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria. Non-disease controls had no history or symptoms of neurologic or psychiatric disorders and were clinically no demented. To minimize the considerable regional differences in pathology, we selected hippocampus in all tested AD patients and relative control.

2.4. Cell culture and treatments

Primary CGC cultures were obtained from 7-day-old Wistar rats as described by Levi et al. (1984). Cells were plated in basal medium Eagle (BME; Invitrogen, Gibco) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin on dishes coated with poly-L-lysine. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18 to 22 h after plating to prevent proliferation of non neuronal cells.

2.5. Cell homogenate preparation

Cultured medium was removed, the plated CGCs were washed and cell homogenate was prepared by scraping cells in PBS, containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO4, 15 mM KH_2PO_4, pH 7.4, followed by homogenization at room temperature by 10 strokes with a Dounce homogenizer. Cytosolic lactate dehydrogenase was released and subsequent treatment with Triton-X-100 did not cause further release. To verify that cell homogenate contains intact mitochondria, the mitochondrial coupling efficiency was checked by measuring the respiratory control index, *i.e.* (oxygen uptake rate after adenosine diphosphate [ADP] addition) / (oxygen uptake rate before ADP addition), which reflects the ability of mitochondria to produce adenosine triphosphate (ATP). As a further control of the intactness of the mitochondrial membranes the lack of activities of adenylate kinase (ADK, E.C.2.7.4.3) and glutamate dehydrogenase (GDH, E.C.1.4.1.3), which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively, was monitored in the postmitochondrial supernatant obtained from cell homogenate after centrifugation at 10,000 rpm for 10 min at 4 °C to create a post-mitochondrial supernatant. Protein content was determined according to Waddell and Hill (1956) with bovine serum albumin used as a standard.

2.6. Isolation of synaptosome-enriched subcellular fractions of brain

Synaptosome-enriched subcellular fractions of hippocampus from human brain were prepared according to (for references see Amadoro et al., 2012). These fractions are largely enriched in both pre- and post-synaptic proteins such as PSD95 and GluR2/3. In brief, human brain was homogenized in 3.5 ml of homogenization buffer (320 mM sucrose/4 mM Hepes, pH7.4/1 mM EGTA/0.4 mM PMSF/plus proteases inhibitor and phosphatase inhibitor cocktail) with 15 strokes of a glass Dounce tissue grinder (Wheaton). The homogenate was centrifuged at 1000 ×*g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the second pellet was resuspended in 2.5 ml of homogenization buffer and centrifuged at 13,000 ×*g* for 15 min. All the steps were performed at 4 °C. The resulting pellet was then resuspended in the same buffer.

2.7. Polarographic measurements

O_2 consumption was measured polarographically by means of a Gilson 5/6 oxygraph using a Clark electrode (for references see Atlante et al., 2003a, 2006). The cell homogenate (about 0.2 mg protein) was incubated in 1.5 ml of the respiration medium (210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM KH_2PO_4/K_2HPO_4, pH 7.4, 3 mM $MgCl_2$) in a thermostated (25 °C) water-jacketed glass vessel and polarographic measurements were performed as substrate-inhibitor analyses (see the figure legend). Instrument sensitivity was such as to allow rates of O_2 uptake as low as 0.5 natom min^{-1} mg^{-1} protein to be followed.

2.8. Safranine O response assay

The safranine O response was monitored as in Atlante et al. (2006, 2008). Time-dependent absorbance changes at 520 nm were recorded with a Jasco double-beam/double-wavelength spectrophotometer UV-550. Measurements were carried out at 25 °C in 2 ml of standard medium consisting of 200 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES-Tris, pH 7.2, containing 1 μM safranine O and 0.1 mg protein cell homogenate.

2.9. Photometric measurement of mitochondrial RC Complex I–V activities

Complex I–V enzymatic activities were assayed photometrically at 25 °C, essentially as in (Bénit et al., 2006; Wharton and Tzagaloff, 1967). Each assay was performed at least in duplicate by using either