Neurobiology of Disease 45 (2012) 417–424

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

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

Mitochondrial dysfunction and accumulation of the β-secretase-cleaved C-terminal fragment of APP in Alzheimer's disease transgenic mice

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ARTICLE INFO

Article history:
Received 4 April 2011
Revised 27 July 2011
Accepted 2 September 2011
Available online 13 September 2011

Keywords:
Alzheimer's disease
Mitochondria
C99
Amyloid precursor protein
β-Secretase
BACE1
Mitochondrial dysfunction
Oxidative DNA damage
Learning and memory
5XFAD

ABSTRACT

Mitochondrial dysfunction is an early feature of Alzheimer's disease (AD) and may play an important role in the pathogenesis of disease. Emerging evidence indicates that amyloid-β (Aβ) peptides enter mitochondria and may thereby disrupt mitochondrial function in brains of AD patients and transgenic model mice. However, it remains to be determined whether the β-cleaved C-terminal fragment (C99), another neurotoxic fragment of amyloid precursor protein (APP), may accumulate in mitochondria of neurons affected by AD. Using immunoblotting, digitonin fractionation and immunofluorescence labeling techniques, we found that C99 is targeted to mitochondria, in particular, to the mitoplast (i.e., inner membrane and matrix compartments) in brains of AD transgenic mice (5XFAD model). Furthermore, full-length APP (fl-APP) was also identified in mitochondrial fractions of 5XFAD mice. Remarkably, partial deletion of the β-site APP-cleaving enzyme 1 ($BACE1^{+/-}$) almost completely abolished mitochondrial targeting of C99 and fl-APP in 5XFAD mice at 6 months of age. However, substantial amounts of C99 and fl-APP accumulation remained in mitochondria of 12-month-old $BACE1^{+/-}$·5XFAD mouse brains. Consistent with these changes in mitochondrial C99/fl-APP levels, $BACE1^{+/-}$ deletion age-dependently rescued mitochondrial dysfunction in 5XFAD mice, as assessed by cytochrome *c* release from mitochondria, reduced redox or complex activities and oxidative DNA damage. Moreover, $BACE1^{+/-}$ deletion also improved memory deficits as tested by the spontaneous alternation Y-maze task in 5XFAD mice at 6 months but not at 12 months of age. Taken together, our findings suggest that mitochondrial accumulation of C99 and fl-APP may occur through BACE1-dependent mechanisms and contribute to inducing mitochondrial dysfunction and cognitive impairments associated with AD.

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease that represents the most common form of dementia among the elderly population. Although the cause of AD has not been completely understood, accumulating evidence indicates that mitochondrial dysfunction and increased oxidative stress occur early in the progression of disease and thus may play an important role in the pathogenesis of AD (Galindo et al., 2010; Moreira et al., 2010; Reddy, 2009; Swerdlow and Khan, 2009). Several in vitro studies have shown that exposure to amyloid-β (Aβ) peptides causes abnormalities of mitochondrial function as characterized by excessive mitochondrial membrane potential depolarization, uncoupling of the mitochondrial respiratory chain, reduced ATP, and increased reactive oxygen species (ROS) generation (Cardoso et al., 2004; Galindo et al., 2010; Reddy and Beal, 2008). Interestingly, cells depleted of mitochondrial DNA are insensitive to Aβ, suggesting that Aβ-mediated neurotoxicity and apoptosis require functional mitochondria (Cardoso et al., 2001, 2002). Moreover, Aβ overproduction induces abnormal mitochondrial dynamics through modulation of mitochondrial fission/fusion proteins (Wang et al., 2008). Consistent with these findings, increased autophagocytosis of mitochondria is indeed a prominent feature of AD (Moreira et al., 2007; Swerdlow and Khan, 2009).

Interestingly, recent investigations indicate that monomeric and oligomeric forms of Aβ are found in mitochondria of brains from AD patients and transgenic model mice, although the precise mechanism by which Aβ peptides are transported to mitochondrial membrane remains unclear (Caspersen et al., 2005; Dragicevic et al., 2010; Du et al., 2010; Hansson Petersen et al., 2008; Manczak et al., 2006). Aβ is formed from amyloid precursor protein (APP) through its endoproteolysis by two enzymes. The β-secretase (called BACE1 for β-site APP-cleaving enzyme 1) cuts APP first to generate the N-terminus of Aβ, producing the intermittent β-cleaved C-terminal fragment (C99). The γ-secretase cleaves C99 subsequently to release Aβ peptides. Importantly, different lines of evidence show that not only Aβ peptides but also C99 fragments may contribute to the pathophysiology of AD such as neurodegeneration, endosome dysfunction, and

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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.nbd.2011.09.001

synaptic or mnemonic deficits (Berger-Sweeney et al., 1999; Choi et al., 2001; Jiang et al., 2010; Lee et al., 2006; Nalbantoglu et al., 1997; Oster-Granite et al., 1996; Song et al., 1998). In contrast to the well-documented interactions between Aβ and mitochondria, it has not been examined whether C99 is targeted to mitochondria and plays a role in inducing mitochondrial dysfunction associated with AD.

In this study, we investigated intramitochondrial accumulation of C99 in brains of the 5XFAD transgenic mouse model of AD, which co-overexpresses human APP and presenilin-1 (PS1) harboring five familial AD (FAD) mutations (Oakley et al., 2006; Ohno et al., 2006; Ohno et al., 2007). We recently demonstrated that partial deletion of the BACE1 gene (BACE1$^{+/-}$) rescues synaptic and memory dysfunctions and prevents neurodegeneration with reductions in cerebral C99 and Aβ levels in relatively earlier pathological stages of 5XFAD mice (6–9 months of age), whereas it is no longer able to affect C99/Aβ levels or exert beneficial effects at advanced age (≥15 months) (Devi and Ohno, 2010a,b; Kimura et al., 2010). These findings raise the possibility that the same degree of β-secretase inhibition may yield less therapeutic benefits as AD progresses into the more profound stage. In this study, we crossed 5XFAD mice with BACE1$^{+/-}$ mice and explored age-related changes in the effect of BACE1$^{+/-}$ reduction on mitochondrial localization of C99 with relevance to improvements in mitochondrial dysfunction and memory deficits in 5XFAD mice.

Materials and methods

Animals

We used 5XFAD transgenic mice (Tg6799 line) that co-express and co-inherit FAD mutant forms of human APP (the Swedish mutation: K670N, M671L; the Florida mutation: I716V; the London mutation: V717I) and PS1 (M146L; L286V) transgenes under transcriptional control of the neuron-specific mouse Thy-1 promoter (Oakley et al., 2006; Ohno et al., 2006, 2007). Hemizygous 5XFAD transgenic mice were crossbred to heterozygous BACE1 knockout (BACE1$^{+/-}$) mice (The Jackson Laboratory, Bar Harbor, ME) (Cai et al., 2001; Laird et al., 2005), yielding animals with four different genotypes (wild-type, BACE1$^{+/-}$, 5XFAD$^{+/-}$, and BACE1$^{+/-}$·5XFAD$^{+/-}$). Genotyping was performed by PCR analysis of tail DNA. All experiments were done blind with respect to the genotype of the mice at 6, 9 or 12 months of age, and were conducted with the approval of the Nathan Kline Institute Animal Care and Use Committee.

Isolation of mitochondria and cytosol fractions

Whole brain samples were taken from the mice under deep isoflurane anesthesia, and isolation of subcellular fractions was performed as described previously (Devi et al., 2006, 2008). Brain tissues were homogenized in 8× volumes of homogenization medium containing 70 mM sucrose, 210 mM mannitol, 2 mM HEPES and 0.1 mM EDTA. Homogenates were centrifuged at 2000×*g* for 10 min. The resultant supernatant was centrifuged at 18,000×*g* for 20 min to obtain the crude mitochondrial pellet, which was layered on 0.8 M sucrose solution and centrifuged at 22,000×*g* for 30 min to remove myelin contamination. The final pellet was resuspended in homogenization medium as mitochondrial fractions. Postmitochondrial supernatant was further centrifuged at 100,000×*g* to obtain cytosolic fractions in supernatant with removal of the microsomal pellet.

Isolation of mitochondria using the Percoll method

The Percoll preparation of mitochondria has been described previously (Barksdale et al., 2010; Brown et al., 2006). Briefly, the brain tissue was placed in a 5× volume of ice-cold homogenization medium and homogenized with a Dounce homogenizer. The resultant homogenate was centrifuged at 1300×*g* for 10 min, and the supernatant was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll, followed by 23% Percoll, and finally the 15% Percoll mitochondrial sample. Samples were centrifuged at 18,000×*g* for 10 min at 4 °C. After centrifugation, the band between 23% and 40% containing nonsynaptic mitochondria was removed from the density gradient and placed in a separate tube. The nonsynaptic mitochondria was washed twice with homogenization medium (10,000×*g* for 15 min), and the resulting pellet was then resuspended in homogenization medium.

Limited trypsin digestion and digitonin fractionation

Part of the mitochondrial samples prepared by the Percoll method (150 μg) was further subjected to treatments with trypsin and digitonin. To remove proteins that are peripherally associated with mitochondria, freshly isolated mitochondria in homogenization medium were subjected to trypsin digestion (30 μg of trypsin per milligram of protein) on ice for 20 min. The reaction was terminated by adding a trypsin inhibitor (300 μg/mg). The mitochondrial suspension was sedimented through 0.8 M sucrose. The final pellet was washed twice with homogenization medium, and the resulting pellet was then resuspended in homogenization medium. A separate set of fresh mitochondrial fractions (150 μg) in homogenization medium was treated with digitonin solution (0.05%) for 10 min with occasional mixing on ice. Digitonin-soluble and -insoluble fractions were separated by centrifugation at 12,000×*g* for 15 min. The pellet was washed and then resuspended in homogenization medium.

Immunoblot analysis

Mitochondrial and cytosolic fractions, as well as whole brain lysates as controls, with added protease inhibitor cocktail (Calbiochem, La Jolla, CA) were mixed with SDS sample buffer. Protein concentrations were determined by a BCA protein assay kit (Pierce, Rockford, IL), and 20–50 μg of protein was run on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. After blocking, membranes were probed with anti-full-length APP (1:1000, 22C11, MAB348, Millipore, Billerica, MA), an antibody that recognizes C-terminal epitope in APP (1:1000, C1/6.1, kindly provided by Dr. Paul Mathews, Nathan Kline Institute) to detect the β-cleaved C-terminal fragment (C99), anti-BACE1 (1:1000, MAB5308, Millipore), anti-synaptophysin (1:5000, ab8049, Abcam, Cambridge, MA), anti-calnexin (1:1000, 610523, BD Biosciences, San Jose, CA), anti-LAMP2 (1:200, ABL-93, Hybridoma Bank, Iowa City, IA), anti-TOM20 (1:1500, sc-11415, Santa Cruz Biotechnology, Santa Cruz, CA), anti-TIM23 (1:2000, sc13298, Santa Cruz Biotechnology), anti-HSP60 (1:5000, ab46798, Abcam), anti-cytochrome *c* (1:5000, MSA06, Mitosciences, Eugene, OR) or anti-β-actin (1:15,000, AC-15, Sigma, St. Louis, MO). They were then incubated with horseradish peroxidase-conjugated secondary IgG. Immunoblot signals were visualized by an ECL chemiluminescence substrate reagent kit (Pierce). Quantitative analysis of band optical density was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Double immunofluorescence labeling

Mice were transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH7.4), followed by 4% paraformaldehyde in PBS under deep isoflurane anesthesia. Brains were post-fixed for 24 h in 4% paraformaldehyde in PBS at 4 °C and transferred to PBS. The brain was sectioned coronally at 30 μm using a vibratome (VT1200, Leica Microsystems, Wetzlar, Germany), and successive sections were stored in PBS containing 0.05% sodium azide at 4 °C. Three sections