Neuroscience Research 72 (2012) 94–102

Contents lists available at SciVerse ScienceDirect

Neuroscience Research

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

Inhibition of mitochondrial permeability transition pore opening is involved in the protective effects of mortalin overexpression against beta-amyloid-induced apoptosis in SH-SY5Y cells

Mingyue Qu, Zhou Zhou*, Chunhai Chen, Min Li, Liping Pei, Ju Yang, Yuan Wang, Li Li, Chuan Liu, Guangbin Zhang, Zhengping Yu, Denggao Wang

Department of Occupational Health, Third Military Medical University, Chongqing 400038, China

ARTICLE INFO

Article history:
Received 26 May 2011
Received in revised form
17 September 2011
Accepted 20 September 2011
Available online 5 October 2011

Keywords:
Alzheimer's disease
β-Amyloid
Apoptosis
Mortalin
Mitochondrial permeability transition
SH-SY5Y cells

ABSTRACT

Mortalin (mtHsp70) is a mitochondrial heat shock protein critical for maintaining the functional integrity of mitochondrial proteins. Our previous study demonstrated that mortalin overexpression protected against Aβ-induced neurotoxicity through a mitochondria-dependent mechanism, but the molecular details remained unclear. Recent biochemical studies implicate opening of the mitochondrial permeability transition pore (mPTP) in Aβ-mediated mitochondrial dysfunction. The present study investigated the effect of mortalin overexpression on Aβ-induced mPTP activation and ensuing neuronal apoptosis. Mortalin overexpression inhibited mPTP activation and protected SH-SY5Y neurons against Aβ-induced apoptosis. Compared to controls, neurons overexpressing mortalin also demonstrated superior intracellular free calcium regulation, lower mitochondrial reactive oxygen species generation, and decreased Bax/Bcl-2 ratios in response to Aβ treatment. Mortalin overexpression suppressed activation of the mitochondrial apoptotic cascade as demonstrated by inhibition of cytochrome c release and caspase-3 activation. Our results indicate that the cytoprotective efficacy of mortalin under Aβ-induced stress is mediated, at least in part, by inhibition of mPTP opening. Demonstration of the neuroprotective action of mortalin provides additional insights into the pathogenic mechanisms of Aβ toxicity and defines possible molecular targets for therapeutic intervention.

1. Introduction

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder characterized by memory loss and other cognitive impairments resulting from neurofibrillary tangles, senile plaques, and neuronal apoptosis. It is widely accepted that amyloid-beta peptide (Aβ), the major component of senile plaques, is toxic to mitochondria, implicating mitochondrial dysfunction in the pathogenesis of Alzheimer's disease (Tillement et al., 2011). Growing evidence indicates that Aβ-mediated mitochondrial dysfunction results from activation of the mitochondrial permeability transition pore (mPTP), a nonselective, high conductance channel spanning the inner and outer mitochondrial membrane (Ferreiro et al., 2008). It is believed that Aβ accumulates in mitochondria and interacts with several mitochondrial proteins that facilitate opening of the mPTP, resulting in increased permeability of mitochondrial membranes and loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) necessary to drive cellular respiration (Ren et al., 2011). Moreover, it is known that Aβ damages neurons by enhancing free radical generation and increasing the intracellular free calcium concentration (Sheehan et al., 1997), both of which are potent inducers of mPTP opening. Uncontrolled mPTP activation results in osmotic swelling, $\Delta\Psi_m$ dissipation, generation of reactive oxygen species (ROS), perturbation of intracellular calcium regulation, uncoupling of the respiratory chain, and release of cytochrome c and other pro-apoptotic factors that eventually initiate apoptosis. These findings point to mPTP activation as a key event in apoptosis and a potential target for cytoprotective intervention.

Mortalin (also known as mtHsp70, PBP74, or GRP75) is a 75 kDa member of the heat shock protein 70 family expressed primarily within mitochondria (Bhattacharyya et al., 1995). As an essential molecular chaperone, mortalin participates in diverse physiological processes, including stress responses, centrosome duplication, intracellular protein trafficking, and cell proliferation (Kaul et al., 2007). Mortalin also facilitates mitochondrial respiration and maintains mitochondrial protein integrity (Liu et al., 2003). Notably, mortalin overexpression has been shown to activate protective mechanisms that allow cell survival under various pathogenic

* Corresponding author at: Department of Occupational Health, The Third Military Medical University, No. 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel.: +86 23 68752289; fax: +86 23 68752289.
E-mail addresses: lunazhou00@163.com, lunazhousci@163.com (Z. Zhou).

0168-0102/$ – see front matter © 2011 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.
doi:10.1016/j.neures.2011.09.009

conditions (Wadhwa et al., 2002). Recent studies have also linked this mitochondrial chaperone to the risk and progression of AD; indeed, mortalin expression is elevated in affected brain regions of both AD patients and animal AD models (David et al., 2006; Osorio et al., 2007). We recently reported that mortalin overexpression attenuated Aβ-induced oxidative damage and ensuing apoptosis in SH-SY5Y cells. Furthermore, inhibition of endogenous mortalin expression makes SH-SY5Y cells more susceptible to Aβ-induced neurotoxicity (Qu et al., 2011). However, the underlying mechanisms remain unclear. The opening of mPTP is a precipitating event in the activation of mitochondrial apoptosis. Moreover, mortalin interacts with the voltage-dependent anion channel (VDAC) component of mPTP, and modifies channel permeability and ion selectivity (Szabadkai et al., 2006). We speculate that inhibition of mPTP is a possible mechanism for the cytoprotective action of mortalin overexpression against Aβ toxicity in SH-SY5Y cells.

To address the role of mPTP inhibition in mortalin neuroprotection, we examined Aβ-induced cellular injury in SH-SY5Y cells overexpressing mortalin. The $A\beta_{25-35}$ peptide used in this study retains the toxicity of the full length Aβ, and is widely used to model Aβ-induced neurodegeneration. Identification of the molecular targets of mortalin could lead to therapeutic interventions against progressive neurodegenerative diseases associated with neuronal apoptosis.

2. Materials and methods

2.1. Cell culture, transfection and Aβ treatment

Human SH-SY5Y neuroblastoma cells (provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium/F12 medium (DMEM/F12, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich). The medium was replaced every 2 days and cultures were maintained in an atmosphere of 5% CO_2 and 95% O_2 humidified air at 37 °C. Human mortalin (GenBank accession number NM_004134) cDNA in the pCMV6 vector was purchased from Origene (Rockville, MD). Cells were transfected with mortalin using the Lipofectamine LTX transfection system (Invitrogen) according to the manufacturer's protocol, and stably transfected colonies were selected in G418 medium (500 μg/ml) as described previously (Qu et al., 2011). Aggregates of Aβ were prepared according to Harada and Sugimoto (1999). Briefly, $A\beta_{25-35}$ (Sigma–Aldrich) was solubilized in distilled water at a concentration of 5 mM, incubated in a capped vial at 37 °C for 3 days to form aggregates, and then stored at −20 °C until use. Immediately before use, these stock solutions were thawed and diluted to the final concentration (25 μM) in medium. Cells were seeded in culture plates, permitted to attach for 24 h and grown to 80% confluence. Upon reaching 75% confluence, cells were washed twice with PBS and then incubated in the fresh medium containing $A\beta_{25-35}$ at 37 °C for 24 h.

2.2. Confocal microscopy with immunofluorescence

For the determination of mortalin expression, SH-SY5Y cells were washed with PBS, fixed with 3.5% paraformaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 for another 10 min (all at room temperature, RT). After two washes with PBS, cells were blocked with PBS supplemented with bovine serum albumin (BSA, 3%) for 30 min at RT. For immunofluorescent staining, cells were incubated with anti-mortalin monoclonal antibody overnight at 4 °C (1:100 dilution; Cell Signaling Technology). After two washes, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:200 dilution; Sigma-Aldrich) at 37 °C in the dark. The nuclei were counterstained with Hoechst 33258. Images of stained cells were captured using a Leica TCS-SP5 confocal laser scanning microscope.

2.3. Western Blot analysis

After washed twice with ice-cold PBS, SH-SY5Y cells were scraped in PBS and collected by centrifugation (300 × *g* for 5 min). The cell pellet was homogenized with lysis buffer (T-PER, Pierce Biotechnology) containing cocktail protease inhibitors (Roche). After 30 min incubation on ice, the lysate was centrifuged at 12,000 × *g* for 20 min, and the supernatant is referred to as the total cellular proteins. Preparation of cytosolic fractions was achieved using a cytosol/mitochondria fraction isolation kit according to the manufacturer's instructions (Sigma–Aldrich). Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

For Western blotting, protein samples (50 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioRad). Membranes were incubated in Odyssey blocking buffer (LICOR) for 1 h at RT and then labeling with a primary antibody overnight at 4 °C. The primary antibodies used for estimation of neural protein expression were rabbit anti-mortalin (1:1000 dilution; Cell Signaling Technology), rabbit anti-Bax (1:1000 dilution; Cell Signaling Technology), rabbit anti-Bcl-2 (1:1000 dilution; Cell Signaling Technology), rabbit anti-cytochrome c (1:2000 dilution; Cell Signaling Technology), and mouse anti-caspase-3 antibodies (1:500 dilution; Cell Signaling Technology). The antibody-tagged membranes were washed three times with TBST and then incubated with IRDye® 800 Donkey Anti-Rabbit antibody (1:5000 dilution; LI-COR) or IRDye® 700 Donkey Anti-Mouse IgG antibody (1:5000 dilution; LI-COR) for 1 h at RT. After final washes in PBS, the fluorescent signals were detected and quantified by the Odyssey infrared imaging system (LI-COR)). To control for gel protein loading, blots were reprobed with a mouse monoclonal anti-β-actin antibody (1:5000 dilution; Sigma–Aldrich).

2.4. Measurement of apoptosis

Apoptotic cell death was analyzed by TUNEL staining using an in situ cell death detection POD kit (Roche Diagnostics Corp). Briefly, 1×10^5 cells/ml were plated onto poly-L-lysine-coated coverslips and incubated for 24 h before treatment. After treatment with $A\beta_{25-35}$ for 24 h, cells were fixed for 20 min with 4% paraformaldehyde in PBS. After rinsing in PBS, apoptotic cells were detected by the TUNEL technique following the manufacturer's protocol. The nuclei were counterstained with Hoechst 33258 and stained cells were observed under a fluorescence microscope (Olympus). The TUNEL-positive cells were counted and the ratio of apoptotic cells to total cells (Hoechst-stained) was determined.

2.5. MTT assay for mitochondrial function and cell viability

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used as an indicator of mitochondrial function and cell viability. This test is based on the reduction of MTT to colored insoluble formazan crystals by mitochondria. The detailed methods were described previously (Xu et al., 2010).

2.6. Detection of opened mitochondrial permeability transition pore (mPTP)

The opening of the mPTP in SH-SY5Y cells was assessed using a calcein–cobalt mPTP assay kit (Genmed Scientifics) according to the manufacturer's instructions and methods described previously (Chen et al., 2009). Briefly, the treated cells were washed