The International Journal of Biochemistry & Cell Biology 41 (2009) 2015–2024

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Alpha-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells

Mordhwaj S. Parihar [a], Arti Parihar [b], Masayo Fujita [c], Makoto Hashimoto [c], Pedram Ghafourifar [d,*]

[a] School of Studies in Biotechnology & Zoology, Vikram University, Ujjain, MP, India
[b] Department of Biological Sciences, GDC College, Vikram University, Ujjain, MP, India
[c] Laboratory for Chemistry and Metabolism, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan
[d] Tri-State Institute of Pharmaceutical Sciences, Huntington, WV, USA

ARTICLE INFO

Article history:
Available online 19 May 2009

Keywords:
Alpha-synuclein
Mitochondria
Oxidative stress
Respiration
Human neuroblastoma cells

ABSTRACT

Overexpression of alpha-synuclein and oxidative stress has been implicated in the neuronal cell death in Parkinson's disease. Alpha-synuclein associates with mitochondria and excessive accumulation of alpha-synuclein causes impairment of mitochondrial functions. However, the mechanism of mitochondrial impairment caused by alpha-synuclein is not fully understood. We recently reported that alpha-synuclein associates with mitochondria and that overexpression of alpha-synuclein causes nitration of mitochondrial proteins and release of cytochrome c from the mitochondria [Parihar M.S., Parihar A., Fujita M., Hashimoto M., Ghafourifar P. Mitochondrial association of alpha-synuclein causes oxidative stress. Cell Mol Life Sci. 2008a;65:1272–1284]. The present study shows that overexpression of alpha-synuclein A53T or A30P mutants or wild-type in human neuroblastoma cells augmented aggregation of alpha-synuclein. Immunoblotting and immuno-gold electron transmission microscopy show localization of alpha-synuclein aggregates within the mitochondria of overexpressing cells. Overexpressing cells show increased mitochondrial reactive oxygen species, increased protein tyrosine nitration, decreased mitochondrial transmembrane potential, and hampered cellular respiration. These findings suggest an important role for mitochondria in cellular responses to alpha-synuclein.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Alpha-synuclein (α-syn) is a major component of intraneuronal inclusions found in familial and sporadic Parkinson's disease (PD), and the glial inclusions in multiple system atrophy (Polymeropoulos et al., 1997; Spillantini et al., 1998; Galvin et al., 2001). The discovery of two point mutations at A53T and A30P on α-syn has been considered a landmark in establishing the role of α-syn in PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). However, lack of α-syn mutations in vast majority of patients with idiopathic (nonfamilial) PD (Chan et al., 1998) along with findings showing that overexpression of wild-type (WT) α-syn induces dopaminergic cell death (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004). These studies indicate that additional mechanisms are required for α-syn to become toxic. Various mechanisms have been suggested for cellular toxicity of α-syn. One such mechanism is the enhanced tendency of α-syn to self-assemble or aggregate (Ibanez et al., 2004; Conway et al., 1998; Trojanowski and Lee, 1998; Narhi et al., 1999) that triggers pathological processes leading to cell death (McNaught and Olanow, 2006; Souza et al., 2000). Aggregated α-syn has been found in disease states associated with augmented oxidative stress resulting in oxidative damage to cellular components. Other mechanisms include interaction of α-syn with membranes (Ding et al., 2002; Smith et al., 2005; Volles and Lansbury, 2007) and increased oxidative stress (Hashimoto et al., 1999). Remarkably, several other proteins involved in neurodegeneration such as amyloid beta peptide (Manczak et al., 2006; Sayre et al., 2008) and mutant huntingtin (Sayre et al., 2008; Choo et al., 2004) also induce oxidative stress. Mitochondria are membranous organelles highly involved in oxidative stress and apoptotic cell death. We recently reported that overexpression of α-syn causes nitration of mitochondrial proteins and release of cytochrome c from the mitochondria of human neuroblastoma cells (Parihar et al., 2008a). The present study was conducted to delineate subcellular localization and mechanisms underlying cellular toxicity caused by α-syn overexpression. Our findings show that α-syn interacts with mitochondria, and that expression of WT or mutants A53T or A30P α-syn augments oxidative stress and suppresses mitochondrial and cellular functions.

* Corresponding author.
E-mail address: Pedram.Ghafourifar@TIPSwv.com (P. Ghafourifar).

1357-2725/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2009.05.008

2. Materials and methods

2.1. Cell culture

Human dopaminergic neuroblastoma SHSY cells obtained from ATCC (Manassas, VA, USA) were grown onto poly-D-lysine-coated 12 mm glass coverslips in 24-well plates or in T25 flasks with DMEM/F-12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C and their viability verified by Trypan Blue exclusion assay.

2.2. α-Syn constructs

A two-step PCR strategy was performed as to create α-syn A53T expression vector as described (Hashimoto et al., 1997). Briefly, the following two sets of primer pairs were individually incubated with pCEP4- α-syn as template in the first PCR reaction: sense primer 5′-ATGGATGTATTCATGAAAGGACT-3′ (the N-terminal 1–23 oligonucleotide position of α-syn) and antisense primer 5′-CTCAGCCA-CTGTTGTCACACCATGCAC-3′ (antisense sequence of 197–223th oligonucleotide position of α-syn with A53T mutation; Polymeropoulos et al., 1997), and sense primer 5′-GTGCATGGTGT-GACAACAGTGGCTGAG-3′ (sense sequence 197– 223th oligonucleotide position of α-syn with A53T mutation) and antisense primer 5′-TTAGGCTTCAGGTTCGTAGTCTTG-3′ (the C-terminal 400–423th oligonucleotide position of α-syn). The PCR products were gel-purified and incubated in the second PCR reaction with the N- and the C-terminal human α-syn primers to synthesize a full-length α-syn A53T cDNA. The resulting PCR products were inserted into mammalian expression vector p-TARGET by using TA cloning (Promega Biotech, Madison, WI). Same two-step PCR was carried out to create α-syn A30P expression vector using the following primers: antisense primer 5′-TTTGTCTTTCCTGGTGCTTCTGCCAC-3′ and sense primer 5′-GTGGCAGAAGCACCAGGAAAGACAAA-3′ (122–148th oligonucleotide position of α-syn with A30P mutation; Kruger et al., 1998). To create WT α-syn expression vector, a regular PCR was performed using N- and C-terminal human α-syn primers and subcloned into the p-TARGET, while a self-ligated plasmid of the p-TARGET was used as control vector. Fidelity of sequences was confirmed for all plasmids.

2.3. Transfection

SHSY cells grown on glass coverslips were transfected with α-syn A53T or A30P mutant, WT, or empty vector at 70–80% confluence using Lipofectamine 2000 (Invitrogen) as we have described (Parihar et al., 2008a).

2.4. Immunofluorescence detection of α-syn aggregation in cells

To determine α-syn aggregates and its subcellular localization, cells were loaded with the mitochondrial marker mitotracker red (Molecular Probes; 500 nM) in DMEM/F12 medium under 5% CO_2 at 37 °C for 30 min, washed three times with phosphate buffer saline (PBS) and fixed in freshly prepared 4% paraformaldehyde for 20 min at 4 °C as described (Parihar et al., 2008a). After three washes in Tris-buffer saline, samples were immunostained overnight with monoclonal antibody against α-syn (H3C; 1:500 dilution) followed by 60-min incubation with an anti-mouse Alexa 488-conjugated secondary antibody (Molecular Probes: 1:200 dilution).

2.5. Detection of mitochondrial reactive oxygen species (ROS) and transmembrane potential ($\Delta\psi$)

Mitochondrial ROS was determined by probing cells with mitotracker red (200 nM) and chloromethyl-2,7-dichlorodihydrofluorescein diacetate (DCF-DA; 15 μM; Molecular Probes) in DMEM/F12 medium under 5% CO_2 at 37 °C for 20 min as described (Parihar et al., 2008b). The $\Delta\psi$ was measured by loading cells with tetramethyl rhodamine methyl ester (TMRM; 10 nM; Molecular Probes) in DMEM/F12 medium under 5% CO_2 at 37 °C for 20 min (Fink et al., 1998). At the end of $\Delta\psi$ measurements, FCCP (5 μM) was used to collapse $\Delta\psi$ as described (Parihar et al., 2008c).

2.6. Immunofluorescence detection of protein tyrosine nitration in cells

To determine mitochondrial protein tyrosine nitration, cells were probed with the mitochondrial marker mitotracker red (500 nM; Molecular Probes) in DMEM/F12 medium under 5% CO_2 at 37 °C for 30 min, washed three times with PBS, and fixed in freshly prepared 4% paraformaldehyde for 20 min at 4 °C. After permeabilizing with triton X-100 (0.2%) and blocking with goat serum (10%), cells were incubated overnight at 4 °C with anti-nitrotyrosine primary antibody (Alexis, 1:250 dilution), followed by 60-min incubation with an anti-mouse Alexa 488 conjugated secondary antibody (Molecular Probes, 1:200 dilution) as described (Parihar et al., 2008c). Cells were air dried and mounted with Vectashield on the glass slide.

2.7. Fluorescent image acquisition and processing

All procedures were conducted as we have described (Parihar et al., 2008a,b,c). Briefly, the coverslips containing probed cells were washed twice with low fluorescence DMEM. Images were acquired using a Zeiss confocal microscope with 63× water objective equipped with LSM 5 software. Fluorescence measurements were performed at room temperature using multichannel detection of excitation with the 488 nm line of an argon laser, and 543 and 633 nm lines of HeNe1 and HeNe2. Fluorescence of mitotracker red and TMRM were acquired using 543 nm excitation and 579 nm emission. Fluorescence of DCF and Alexa 488 were acquired using 488 nm excitation and 516 nm emission. Images were acquired at 12-bit resolution by taking single z-stack 1 μm steps. Fluorescence intensity was quantitated using ImageJ (NIH) as described (Parihar et al., 2008a,c).

2.8. Oxygen consumption

Cells were suspended at 10^7 cells/ml and oxygen consumption was measured using a Clark-type oxygen electrode (Harvard Apparatus, Holliston, MA) as described (Clementi et al., 1998; Ghafourifar et al., 1999a).

2.9. Transmission electron microscopy

Subcellular localization of α-syn was tested by immuno-gold electron microscopy as described (Parihar et al., 2008a). In brief, SHSY cells grown on 22 mm coverslips in 6-well plates were transfected and fixed in 4% paraformaldehyde and 0.04% glutaraldehyde in 0.1 M PBS (pH 7.4) for 3 h at 4 °C. Cells were post-fixed with 0.05% osmium tetroxide 15 min at 4 °C, dehydrated with graded acetone and embedded in spurr resin (Agar Scientific, Stansted, Essex, UK). Following polymerization, ultra-thin sections of 80 nm were prepared using ultramicrotome (Leica Microsystems Inc.). Sections were permeabilized on Formvar coated nickel grids with aqueous 10% hydrogen peroxide for 15 min, washed thrice 5 min each in D-PBS (Gibco), and exposed from 30 min to 5% heat-treated normal goat serum in PBS (Electron microscopy science, Hatfield, PA). Sections were incubated overnight at 4 °C with primary α-syn antibody (H3C; 1:100 dilution) followed by incubation in gold-labeled rabbit anti-mouse IgG (Electron microscopy science, Hatfield, PA).

Download English Version:

https://daneshyari.com/en/article/1984078

Download Persian Version:

https://daneshyari.com/article/1984078

Daneshyari.com