Neurochemistry International 62 (2013) 703–711

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

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

Review

Raised calcium and oxidative stress cooperatively promote alpha-synuclein aggregate formation

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ARTICLE INFO

Article history:
Available online 15 November 2012

Keywords:
Alpha-synuclein
Oxidative stress
Calcium
Parkinson's disease

ABSTRACT

Cell loss in Parkinson's and Parkinson's-plus diseases is linked to abnormal, aggregated forms of the cytoplasmic protein, α-synuclein (α-syn). The factors causing α-syn aggregation may include oxidative stress, changes in protein turnover and dysregulation of calcium homeostasis, resulting in cytotoxic aggregated α-syn species. Recently, we showed that raised calcium can promote α-syn aggregation. We have now investigated the effects of raised calcium combined with oxidation/oxidative stress on α-syn aggregation both *in vitro* and *in vivo*. We treated monomeric α-syn with calcium, hydrogen peroxide or calcium plus hydrogen peroxide *in vitro* and used size exclusion chromatography, fluorescence correlation spectroscopy, atomic force microscopy and scanning electron microscopy to investigate protein aggregation. Our *in vitro* data is consistent with a cooperative interaction between calcium and oxidation resulting in α-syn oligomers. In cell culture experiments, we used thapsigargin or ionophore A23187 to induce transient increases of intracellular free calcium in human 1321N1 cells expressing an α-syn-GFP construct both with and without co-treatment with hydrogen peroxide and observed α-syn aggregation by fluorescence microscopy. Our *in vivo* cell culture data shows that either transient increase in intracellular free calcium or hydrogen peroxide treatment individually were able to induce significantly ($P = 0.01$) increased 1–4 μm cytoplasmic α-syn aggregates after 12 h in cells transiently transfected with α-syn-GFP. There was a greater proportion of cells positive for aggregates when both raised calcium and oxidative stress were combined, with a significantly increased proportion ($P = 0.001$) of cells with multiple (3 or more) discrete α-syn focal accumulations per cell in the combined treatment compared to raised calcium only. Our data indicates that calcium and oxidation/oxidative stress can cooperatively promote α-syn aggregation both *in vitro* and *in vivo* and suggests that oxidative stress may play an important role in the calcium-dependent aggregation mechanism.

1. Introduction

The neuropathology of Parkinson's disease (PD) and the Parkinson's-plus diseases, such as Multiple System Atrophy (MSA), is characterised by microscopically visible cytoplasmic aggregates, or inclusion bodies, within neural cells composed largely of the aggregation-prone protein, α-synuclein (α-syn) (Jellinger, 2009; Stefanova et al., 2009; Vekrellis et al., 2011; Marques and Outeiro, 2012). The formation of inclusion bodies is widely believed to be part of a protective mechanism involving aggresome formation whereby potentially cytotoxic soluble α-syn oligomers are sequestered from the cell (Cookson, 2009; Kim and Lee, 2008; Pountney et al., 2005). A wide range of *in vitro* and *in vivo* data strongly suggests a central role of α-syn molecular interactions in the pathogenesis of synucleinopathies (Polymeropoulos et al., 1997; Lesage and Brice, 2009). Thus, A53T, E46K and A30P mutations linked to rare familial PD forms confer an increased rate of α-syn oligomerisation, although only the A53T and E46K mutants show a significant increase in the rate of fibril formation compared to wild-type (Conway et al., 2000; Fredenburg et al., 2007). In mice and drosophila, over-expression of α-syn has been attributed to the formation of aggregates and neurotoxicity (Fernagut and Chesselet, 2004; Feany and Bender, 2000). Moreover, cell lines transfected with α-syn (wild-type or mutants) form cytoplasmic aggregates positive for α-syn (Bodner et al., 2006). The normal cellular function of α-syn is still not fully elucidated, although it has been implicated in neurotransmitter vesicle binding and recycling (Nemani et al., 2010). Monomeric α-syn is a natively unfolded protein in dilute solution (Uversky, 2009), whilst interaction with lipid vesicles results in folding yielding a more ordered structure (Uversky and Eliezer, 2009; Perlmutter et al., 2009; Drescher et al., 2010). Many

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http://dx.doi.org/10.1016/j.neuint.2012.11.004

different exogenous factors, including oxidation, metal ions and lipids, have been shown to induce the formation of potentially cytotoxic soluble α-syn oligomers (Breydo et al., 2012).

Furthermore, although ultrastructural analysis has revealed that pathological α-syn inclusion bodies, i.e. Lewy bodies, are composed of 9–10 nm amyloid-like filaments analogous to filaments that can be formed *in vitro* from recombinant α-syn (Shults, 2006) recombinant α-syn forms a variety of oligomeric species with diverse structures (spherical, annular, rod-like) under different *in vitro* conditions (Lashuel et al., 2002). Indeed, annular α-syn oligomers that structurally resemble bacterial pore-forming toxins have been demonstrated that can permeabilize lipid vesicles, and are a prime candidate for the key toxic species (Volles and Lansbury, 2003; Danzer et al., 2007; Schmidt et al., 2012).

In previous studies, we showed that calcium binding associated with C-terminal domain can selectively accelerate the formation of the potentially cytotoxic annular α-syn oligomers (Lowe et al., 2004) and more recently that calcium addition to recombinant α-syn monomer *in vitro* causes the dose-dependent formation of oligomers (Nath et al., 2011). Furthermore, we demonstrated that increased intracellular free calcium causes the formation of microscopically-visible α-syn aggregates in the cytoplasm of α-syn-eGFP-transfected human glioma cells following treatment with either thapsigargin or calcium ionophore to induce transiently raised free calcium (Nath et al., 2011). Here, we have investigated the interaction between oxidation/oxidative stress and raised calcium and have found that these two factors act cooperatively to induce α-syn aggregates.

2. Materials and methods

2.1. Chromatography of recombinant α-syn

Recombinant human monomeric α-syn was expressed in an *Escherichia coli* protein expression strain transformed with the human α-syn and the T7 lysozyme cDNA sequences, as reported (Lowe et al., 2004). Cellular debris was pelleted and the supernatant removed and incubated at 95 °C for 5 min, then supernatant was removed and stored at −20 °C. Lysis buffer contained 5 mM EDTA to remove any adventitious metal ions. α-Syn was purified using a HiPrepTM 16/10 Q FF (GE Healthcare) anion exchange column, and the peak fraction was then further purified by gel permeation chromatography (SuperdexTM 75 10/300 GL; GE Healthcare) in 20 mM Tris–HCl, 0.15 M NaCl (pH 7.4) to separate the monomeric species from any oligomeric species present. The peak fractions were either used immediately or snap frozen in liquid nitrogen and stored at −20 °C prior to use. For size exclusion analysis, 10 μM α-syn aliquots were incubated overnight at 4 °C either with 100 μM calcium only or 1 mM H_2O_2 only, combined Ca/H_2O_2 treatment or control, and then separated by gel permeation chromatography as above.

2.2. Scanning electron and atomic force microscopy

The micro-architecture of α-syn species was observed using scanning electron microscopy (SEM). Snap frozen samples were thawed on ice, then absorbed onto cellulose ultrafiltration membrane and washed with ultrapure water to remove any salts present from the buffer. Samples were gold coated and imaged using an FEI Quanta 200 Scanning electron microscope running Tm 2.01. AFM images were taken of samples adsorbed onto freshly-cleaved mica using a NT-MDT p47 Solver scanning probe microscope in semi-contact mode using a silicon cantilever. Height measurements of the particles were taken from a horizontal cross section through the middle of the particle on the reconstructed height image.

2.3. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) measurements were performed on an LSM510/ConfoCor II combination (Zeiss, Jena, Germany). For FCS analysis, N-terminal Alexa-568 labelling was via the amino terminus as described (Nath et al., 2010). This was done either on control, untreated α-syn or after treatment at 4 °C overnight with 100 μM calcium and 1 mM H_2O_2. Samples were freeze-dried for storage and rehydrated immediately prior to measurement. Experiments were done at a concentration of ~10 nM post-labelled protein (N-Alexa-SYN) to ensure that the number of fluorescent molecules in the confocal volume (0.312 fl) is limited to ~5.

2.4. Fluorescence microscopy

Laser confocal images were acquired using an Olympus FV1000 microscope under 60× oil immersion objective. Each image is a compressed Z-stack (10 steps) through the cell. Inclusion bodies were observed as a focal fluorescence signal that was distinctly higher in intensity than the background (cytosolic α-syn) in one or more optical slice.

2.5. Cell culture

1321N1 glioma cells were transiently transfected with a mammalian expression vector containing an α-syn-GFP fusion protein. Cells were seeded at 20,000 cells/well on glass cover slips in 24-well plates and allowed to recover in DMEM and 10% FBS. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with the C-terminal α-syn-GFP fusion construct (McLean et al., 2001), and allowed 18 h to express the protein. Intracellular free calcium was increased using either 1 μM Thapsigargin (Sigma) or 1 μM ionophore A23187 (Sigma) as previously (Nath et al., 2011). Hydrogen peroxide was added to cultures at 10 μM. Cell viability was determined of the treated compared to untreated cells by crystal violet assay. Cell morphology was assessed by differential interference contrast microscopy. Cells showed no evidence of clumping or nuclear fragmentation. Based on DAPI nuclear stain, apoptotic nuclei represented <5% for each treatment condition. For confocal fluorescence microscopy, cells were methanol/acetone fixed and mounted in Prolong Gold mounting medium containing DAPI nuclear stain (Invitrogen).

2.6. Cell counting

The total numbers of α-syn inclusion bodies were counted per cell from the confocal imaging data for each cell treatment. α-Syn aggregation was scored as the percentage of transfected cells with 1–10 discrete visible aggregates. Each experimental condition was repeated in triplicate, with at least 80 transfected cells scored in a blinded manner. The average of three separate experiments is shown in each case, for fixed cells. Error bars are standard deviation.

2.7. Statistical methods

Cell counting data was analysed pair-wise between treatment groups by two-tailed, paired tests t-test using either the Excel or Origin software.

3. Results

Oxidative stress has long been implicated as a causative factor in neurodegenerative disease development and α-syn aggregation