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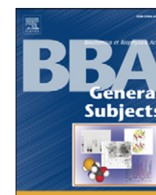

Cellular response of human neuroblastoma cells to α-synuclein fibrils, the main constituent of Lewy bodies

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ABSTRACT

Background: α-Synuclein (α-Syn) fibrils are the main constituent of Lewy bodies and a neuropathological hallmark of Parkinson's disease (PD). The propagation of α-Syn assemblies from cell to cell suggests that they are involved in PD progression. We previously showed that α-Syn fibrils are toxic because of their ability to bind and permeabilize cell membranes. Here, we document the cellular response in terms of proteome changes of SH-SY5Y cells exposed to exogenous α-Syn fibrils.
Methods: We compare the proteomes of cells of neuronal origin exposed or not either to oligomeric or fibrillar α-Syn using two dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry.
Results: Only α-Syn fibrils induce significant changes in the proteome of SH-SY5Y cells. In addition to proteins associated to apoptosis and toxicity, or proteins previously linked to neurodegenerative diseases, we report an overexpression of proteins involved in intracellular vesicle trafficking. We also report a remarkable increase in fibrillar α-Syn heterogeneity, mainly due to C-terminal truncations.
Conclusions: Our results show that cells of neuronal origin adapt their proteome to exogenous α-Syn fibrils and actively modify those assemblies.
General significance: Cells of neuronal origin adapt their proteome to exogenous toxic α-Syn fibrils and actively modify those assemblies. Our results bring insights into the cellular response and clearance events the cells implement to face the propagation of α-Syn assemblies associated to pathology.
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1. Introduction

α-Synuclein (α-Syn) is a 140-residue abundant cytosolic neuronal protein enriched in synaptic terminals where it has been reported to be involved in synaptic vesicle trafficking [1], regulation of synaptic vesicle pool [2], and assembly of a SNARE-complex [3]. Under pathological conditions, α-Syn aggregates into fibrillar assemblies that are the major component of Lewy bodies (LBs), the hallmarks of Parkinson's disease (PD), Multiple System Atrophy (MSA) and Dementia with Lewy Bodies (DLBs).

The finding that LBs in the brains of individuals developing PD contaminate grafted fetal mesencephalic progenitor neurons decades after transplantation [4,5] led to the notion that fibrillar α-Syn propagates within the brain. Evidences for propagation came from the observation that the injection of brain extracts and LBs-enriched fractions from PD patients into the brain of non-human primates, wild-type and model mice induces the formation of lesions characteristic of PD several months after delivery [6–10]. Also, the finding that exogenous α-Syn fibrils are taken up and spread from one cell to another in cell cultures further strengthened this notion [11–17].

The cytotoxicity of extracellular α-Syn in its fibrillar [18,19] or monomeric/oligomeric [20] forms was reported. α-Syn fibrils are toxic to cells because of their ability to bind and permeabilize cell membranes [21]. To assess the changes due to the presence of LBs and associated neurodegeneration in the substantia nigra, the proteomes of post-mortem brain samples from PD patients and healthy individuals were compared [22,23]. Increased levels of proteins involved in oxidative stress, in the protective anti-oxidant system and associated with impaired mitochondrial function were reported. These changes do not reflect necessarily the early response of healthy neuronal cells to exposure to α-Syn fibrils.

The clearance of extracellular α-Syn by several intra- and extracellular proteases has also been documented. Neurosin cleaves soluble α-Syn predominantly in its central part, slowing down its aggregation and decreasing its toxicity [24,25]. The matrix metalloprotease MMP3

Abbreviations: α-Syn, α-Synuclein; 2D-DIGE, two dimensional differential in-gel electrophoresis; MS, mass spectrometry; PD, Parkinson's disease; PTMs, post-translational modifications; LBs, Lewy bodies; PD, Parkinson's disease; DLBs, Dementia with Lewy Bodies; emPAI, Exponentially Modified Protein Abundance Index.

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cleaves the C-terminal region and the central part of α-Syn yielding short fragments that increase α-Syn aggregation [26]. Plasmin mainly cleaves the N-terminal region of extracellular α-Syn inhibiting its translocation into neighboring cells [27] while the lysosomal cathepsins D and B generate C-terminally truncated α-Syn [28,29] and the calcium-regulated intracytoplasmic calpain I cleaves α-Syn within its N-terminal, central or C-terminal regions [30,31]. Besides being truncated, predominantly within the 20 to 25 C-terminal amino-acids [32], α-Syn isolated from LBs of patients with synucleinopathies is post-translationally modified by phosphorylation [32,33], ubiquitination [32,34], N-terminal acetylation [32], nitrosylation and oxidation [35,36]. Whereas most of these post-translational modifications (PTMs) have been shown to favor α-Syn assembly into fibrils, phosphorylation of Ser 129 is considered as one of the main disease-associated α-Syn PTMs significantly up-regulated under pathological conditions. It is therefore used as a marker of disease [32,33].

We previously showed that fibrillar α-Syn is more toxic than its soluble oligomeric precursors upon 24 h exposure of human catecholaminergic neuroblastoma SH-SY5Y cells, a neuronal cell model in PD research [37], to equal particle concentrations of fibrillar and soluble on-assembly pathway oligomeric α-Syn [21]. Here we document the changes in the proteome of these cells of neuronal origin following a similar 24 h exposure to exogenous α-Syn assemblies and characterize cell-mediated exogenous α-Syn PTMs using two dimensional differential in-gel electrophoresis (2D-DIGE). Exogenous oligomeric α-Syn did not induce significant changes within the proteome of SH-SY5Y cells while fibrillar α-Syn did. Proteins which expression increases or decreases were identified by mass spectrometry (MS). In addition to that of proteins involved in apoptosis (TGF, PP2A, LZIC and sorcin) or linked to neurodegenerative diseases (ApoA1, ApoE and RPSA), the expression of Rab6A and VPS28 involved in vesicle trafficking was up-regulated in SH-SY5Y cells exposed to exogenous α-Syn fibrils. Moreover, we observed that exogenous fibrillar, not oligomeric, α-Syn is heavily post-translationally modified, mostly through C-terminal truncations and N-terminal acetylation. Only low levels of ubiquitinylation were detected and no significant changes in phosphorylation and nitrosylation levels were observed. These truncations contribute to increased α-Syn molecular heterogeneity and certainly reflect strategies that the cells implement to counteract the deleterious properties of α-Syn fibrils.

2. Materials and methods

2.1. Production of α-Syn oligomers and fibrils

Recombinant human wild-type α-Syn was expressed and purified as reported previously [38]. α-Syn concentration was determined spectrophotometrically using an extinction coefficient of 5960 M^{-1} cm^{-1} at 280 nm. Pure α-Syn (0.5–1 mM) in 50 mM Tris–HCl, pH 7.5, 150 mM KCl (assembly buffer) was filtered through sterile 0.22 μm filters and stored at −80 °C.

α-Syn soluble oligomers were prepared by incubating monomeric α-Syn in assembly buffer at 4 °C without shaking for 7 days. Oligomeric α-Syn was separated from monomeric α-Syn by size exclusion chromatography using a Superose6 HR10/300 column (GE Healthcare) equilibrated in phosphate buffered saline (PBS) buffer. α-Syn fibrils were produced by incubation of 200 μM monomeric α-Syn in assembly buffer at 37 °C for 4 days under continuous shaking in a thermomixer (Eppendorf, Germany) set at 600 rpm. Fibrils homogeneous in size were obtained by sonication for 20 min on ice in 2-ml Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 W, 2 4 kHz; Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 s pulses as previously described [21]. The nature of α-Syn assemblies was assessed with a Jeol 1400 Transmission Electron Microscope (Jeol) and by analytical ultracentrifugation as described [21]. The particle concentration of α-Syn oligomers and fibrils was obtained by dividing α-Syn monomeric concentration by the average number of monomeric units composing oligomers or fibrils (as measured by analytical ultracentrifugation). For our preparation, the average number of molecules measured for soluble oligomeric and fibrillar α-Syn was 40 and 8300, respectively. Thus, at a working equivalent monomer concentration of 10 μM, the overall particle concentration of soluble oligomeric (sOl) or fibrillar (F) α-Syn was ~0.25 μM and 1.2 nM respectively [21]).

2.2. Exposure of SH-SY5Y cells to α-Syn oligomers and fibrils

Human neuroblastoma SH-SY5Y cells (ECACC; Sigma Aldrich, St. Louis, MO, USA) were grown on 60 cm^2 Petri dishes at 37 °C in humidified air with 5% CO_2 in Dulbecco's modified Eagle Medium/Ham's nutrient mixture F12 (PAA Laboratories GmbH, Pasching, Austria) containing 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells at 30% confluence were exposed for 24 h to either soluble oligomeric (sOl) or fibrillar (F) α-Syn (10 μM equivalent monomer concentration). Untreated cells were used as controls (C). Then, the cells were rapidly washed twice in PBS at 37 °C and lifted with 0.25% trypsin/0.02% EDTA (Sigma) (2 min treatment, rapidly stopped by addition of an excess of fresh medium). Cells were then pelleted by a 3 min centrifugation at 180 *g* and washed twice with 10 ml TBS at 37 °C pH 7.4. Dry pellets were flash-frozen in liquid nitrogen and stored at −80 °C until processing for 2D electrophoresis.

2.3. Sample preparation for 2D-electrophoresis

3×10^6 cells were lysed and homogenized 20 min on ice in 100 μl of UTCD buffer (8 M urea, 2 M thiourea, 4% CHAPS and 50 mM dithiothreitol (DTT)). The samples were treated with 1% Nuclease mix (GE Healthcare) to remove all traces of nucleic acids. The lysates were centrifuged at 20,000 *g* and 4 °C for 1 h. The supernatants were collected, and proteins were precipitated with a 2-D Clean-Up Kit (GE Healthcare) following the manufacturer's instructions. The pellets were solubilized in 100 μl of UTC buffer (UTCD buffer without DTT), and the protein concentration determined using Quick-Start Bradford Dye Reagent (Bio-Rad).

2.4. Two dimensional-differential in-gel electrophoresis (2D-DIGE)

Five independent samples for each condition [control cells (C1–C5); cells exposed to soluble oligomeric α-Syn (sOl1–sOl5 = S1–S5); cells exposed to α-Syn fibrils (F1–F5)] were analyzed by 2D-DIGE. 50 μg of proteins for each sample were labeled with Cy3 or Cy5 CyDyes™ Fluor minimal dyes (GE Healthcare) following the manufacturer's instructions. The internal standard (IS = Std) was prepared by mixing equal amounts of each sample and labeled with Cy2. 50 μg of labeled samples (Cy3 or Cy5) and internal standard (Cy2) were mixed in eight different combinations as follows: C1-Cy3/S3-Cy5/IS-Cy2, F1-Cy3/C4-Cy5/IS-Cy2, S1-Cy3/F4-Cy5/IS-Cy2, F5-Cy3/C2-Cy5/IS-Cy2, S5-Cy3/F2-Cy5/IS-Cy2, C5-Cy3/S2-Cy5/IS-Cy2, C3-Cy3/S4-Cy5/IS-Cy2, and F3-Cy3/C5-Cy5/IS-Cy2. Each of the eight mixes (150 μg) was analyzed by 2D-DIGE as previously described with minor modifications [39]. Protein separation was performed by isoelectrofocusing on 18-cm pH 4–7 Immobiline™ Drystrips (IPG strips, GE Healthcare) in the first dimension and SDS-PAGE on eight different 8 to 18% acrylamide gels (G01–G08) in the second dimension. Cy2, Cy3, and Cy5 components of each gel were individually imaged as described previously [39].

2.5. Statistical analysis

Spot detection, relative quantification of spot intensity, and statistical evaluation using the Student's *t*-test *p*-value were carried out with DeCyder 7.0 software (GE Healthcare). Normalization across all gels was performed using the internal standard.

A spot was considered as differentially represented between two sample groups if the following conditions were fulfilled: protein abundance

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