Nitration inhibits fibrillation of human α-synuclein in vitro by formation of soluble oligomers

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Abstract The aggregation of α-synuclein in dopaminergic neurons is a critical factor in the etiology of Parkinson's disease (PD). Oxidative and nitrative stress is also implicated in PD. We examined the effect of nitration on the propensity of α-synuclein to fibrillate in vitro. Fibril formation of α-synuclein was completely inhibited by nitration, due to the formation of stable soluble oligomers (apparently octamers). More importantly the presence of sub-stoichiometric concentrations of nitrated α-synuclein led to inhibition of fibrillation of non-modified α-synuclein. These observations suggest that nitration of soluble α-synuclein may be a protective factor in PD, rather than a causative one.

Key words: Parkinson's disease; α-Synuclein; Oxidative stress; Nitration; Fibrillation

1. Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder that affects approximately one million people in the USA. PD symptoms are attributed to the progressive loss of dopaminergic neurons from the substantia nigra region of the brain. Some surviving nigral dopaminergic neurons contain cytosolic filamentous inclusions known as Lewy bodies (LBs) and Lewy neurites [1,2]. Substantial evidence indicates the involvement of α-synuclein in the pathogenesis of PD. For example, fibrillar α-synuclein is a major component of LBs [3], and the A53T and A30P mutations in α-synuclein have been identified in familial early-onset PD [4,5]. The production of α-synuclein in transgenic mice [6] or in transgenic flies [7] leads to motor deficits and neuronal inclusions reminiscent of PD.

α-Synuclein is a small (14 kDa), abundant, presynaptic protein [8,9]. α-Synuclein has four tyrosines, Tyr39, Tyr125, Tyr132 and Tyr135. Structurally, purified α-synuclein belongs to the rapidly growing family of intrinsically unstructured (natively unfolded) proteins [10–19], which have little or no ordered structure under physiological conditions, due to a unique combination of low overall hydrophobicity and large net charge [20]. In vitro, α-synuclein forms fibrils with morphologies and staining characteristics similar to those extracted from disease-affected brain [21–26].

Oxidative injury has been implicated in the pathogenesis of PD [27,28]. Nitration of tyrosine residues in proteins arises from the action of oxygen and nitric oxide and their products, especially peroxynitrite. The existence of extensive and widespread accumulation of nitrated α-synuclein (i.e. protein containing the product of tyrosine nitration, 3-nitrotyrosine) in the LBs from patients with PD, dementia with LB, the LB variant of Alzheimer's disease, and multiple system atrophy has been demonstrated using antibodies to specific nitrated tyrosine residues in α-synuclein [27,28]. It has been concluded that the selective and specific nitration of α-synuclein in these disorders may directly link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies [27]. In addition, the exposure of α-synuclein to peroxynitrite in vitro or in cell cultures leads to both nitration of all four tyrosines of α-synuclein and dityrosine cross-linking, as well as oligomerization of α-synuclein [29,30].

Little is known about the detailed effects of tyrosine nitration on the fibrillation of α-synuclein, although recent investigations suggest that it is the formation of intermolecular dityrosine cross-links that is responsible for the enhanced α-synuclein association [29,31]. Here, we describe the effect of nitration on the fibrillation properties of human recombinant α-synuclein in vitro, and its effect on the fibrillation of normal α-synuclein. Surprisingly, nitration of α-synuclein leads to inhibition of fibrillation.

2. Materials and methods

2.1. Expression and purification of human α-synuclein

Human wild type α-synuclein was expressed using *Escherichia coli* BL21 (DE3) cell line transfected with pRK172/α-synuclein wild type plasmid (generously provided by M. Goedert, MRC, Cambridge, UK). The protein was purified according to the published procedure [21,32].

2.2. Supplies and chemicals

Thioflavin T (ThT) was obtained from Sigma (St. Louis, MO, USA), tetranitromethane (TNM) from Aldrich. All other chemicals were of analytic grade from Fisher. All buffers and solutions were prepared with nanopure water and stored in plastic vials.

2.3. UV absorbance spectroscopy

UV absorbance spectra were determined with semimicro quartz cuvettes (Hellma) with a 5 mm light path using a UV-2401 spectrophotometer (Shimadzu, Japan). Protein quantification was determined using absorption maxima of 275 for control and 428 nm for nitrated α-synuclein. The concentration of nitrated protein was calculated according to [33].

2.4. Oxidation of α-synuclein by TNM

Nitrated α-synuclein was prepared by adding a 50 μl aliquot of 1%

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TNM in ethanol to 500 μl of 4–5 mg/ml protein solution. The reaction mixture was stirred vigorously at room temperature for 10 min and then another 50 μl aliquot of 1% TNM solution was added [34]. After 10 min of stirring the mixture was dialyzed with four changes of appropriate buffer at pH 7.8 to completely remove unreacted TNM. Success of oxidation was confirmed by mass spectrometry (MS) analysis (MicroMass Quattro II). Samples for MS analysis were prepared by diluting 2 μl of protein solution in 200 μl of 50% acetonitrile/50% pH 2.0 HCl.

2.5. Fibril formation assay

Fibril formation of oxidized and non-oxidized α-synuclein was monitored in a fluorescence plate reader (Fluoroskan Ascent). Protein solutions contained 20 μM ThT and 1.0 mg/ml (70 μM) α-synuclein in 40 mM phosphate/100 mM NaCl buffer at pH 7.5. We have previously shown that the presence of ThT does not affect the kinetics of fibrillation. Samples were run in quadruplicate or quintuplicate at 37°C with shaking as described previously [11].

2.6. Fluorescence measurements

Fluorescence measurements were determined using a FluoroMax-3 spectrofluorometer (Instruments S.A., Jobin Yvon Horiba, France) with a 1 ml quartz cuvette (Hellma) and 1 cm excitation pathlength.

2.7. Estimation of hydrodynamic dimensions

Stokes radii were determined using a DynaPro Molecular Sizing Instrument (Protein Solutions, Lakewood, NJ, USA) at α-synuclein concentration of 0.1 mg/ml using a 1.5 mm pathlength 12 μl quartz cuvette. Prior to measurement, solutions were filtered with a 0.1 μm Whatman Anodisc-13 filter. Size-exclusion chromatography high performance liquid chromatography (SEC HPLC) analysis was performed with a TSK-GEL G2000SW$_{XL}$ size-exclusion column using a Waters 2695 system (Waters, Milford, MA, USA) and a Wyatt Mini-DAWN dynamic light scattering and RI detector.

2.8. Lowry assay

Aliquots of 50 μl sample were centrifuged at 13 000 rpm for 20 min. Supernatant was removed and mixed with 50 μl of buffer while the pellet was resuspended in 100 μl of buffer for each sample tested. Supernatant and resuspended pellet were added separately to a mixture of 2 ml reagent A and 0.2 ml 50% Folin reagent [35]. After vigorous mixing, samples were allowed to sit for 2 h to complete the reaction.

2.9. Electron microscopy

Negatively stained electron microscope images were taken using Formvar/carbon grids (Ted Pella, Redding, CA, USA). Grids were prepared by incubation of two-fold diluted protein samples for 10 min, followed by washing three times with water, negative staining with uranyl acetate for 5 min, and then washing with water.

3. Results

3.1. In vitro nitration of human α-synuclein

Electrospray ionization MS analysis of α-synuclein prior to the oxidative modification gave a molecular mass of 14 460 ± 3 Da, which is in agreement with the calculated value based on the amino acid sequence (14 460 Da). The conditions used for the in vitro modification of α-synuclein result in nitration of all four tyrosines, as evidenced by the MS peak positioned at 14 642 ± 3 Da, which corresponds to the mass of human α-synuclein with four nitrated tyrosine groups (14 640 Da).

3.2. Nitration of α-synuclein inhibits fibrillation

ThT is a fluorescent dye that interacts with amyloid fibrils leading to an increase in fluorescence intensity in the vicinity of 480 nm [36]. Fig. 1 compares fibrillation patterns of non-modified and nitrated α-synuclein monitored by ThT fluorescence. Fibril formation for the non-oxidized α-synuclein at neutral pH is characterized by a typical sigmoidal curve, reflecting nucleated polymerization. In contrast, there was no

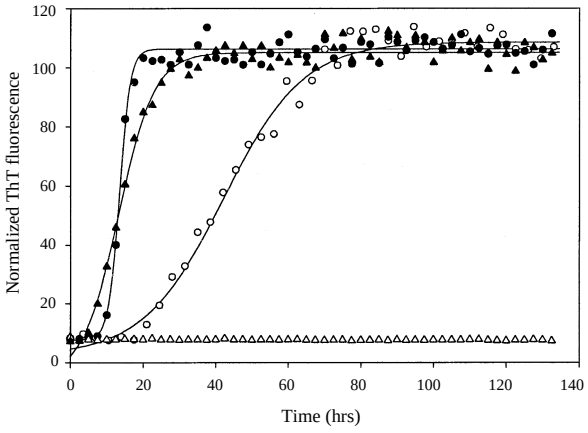

Fig. 1. Nitration of α-synuclein inhibits fibrillation. Kinetics of fibrillation of non-modified (circles) and nitrated α-synuclein (triangles) monitored by ThT fluorescence intensity. Measurements were performed at 37°C and pH 7.5 (open symbols) or pH 3.0 (black symbols) in 20 mM buffer containing 100 mM NaCl with 70 μM α-synuclein.

evidence of fibril formation by nitrated α-synuclein at neutral pH on the time-scale shown. In fact, fibrils were not formed even after incubation of modified α-synuclein for 300 h.

After incubation at pH 7.5, 37°C for 300 h with stirring, the solutions of non-modified and modified α-synuclein were subjected to centrifugation and subsequent analysis of supernatants and pellets by the Lowry protein assay. Results of this analysis are shown in Fig. 2A as relative amount of soluble and insoluble protein in each sample. Fig. 2A shows that most of the non-modified protein was insoluble, whereas the vast majority of the nitrated α-synuclein was in the supernatant, i.e. soluble. This observation shows that nitrated α-synuclein forms neither fibrils, nor amorphous aggregates.

In agreement with earlier studies [11,17,37], incubation of non-modified α-synuclein solution at low pH leads to significant acceleration of the fibrillation process (Fig. 1). This may be attributed to the pH-induced stabilization of a partially folded intermediate, which represents a crucial primary step in the α-synuclein fibrillation pathway [11]. Fig. 1 shows that both non-nitrated and nitrated forms of α-synuclein fibrillate at similar rates at low pH. This means that the inhibitory effect of nitration is eliminated under conditions favoring formation of the critical partially folded intermediate. Fig. 2B represents the results of Lowry assays, which confirm that non-nitrated and nitrated α-synucleins both form mostly insoluble deposits (i.e. fibrils) at acidic pH.

3.3. Nitrated α-synuclein forms stable oligomers

In order to obtain more information about the association state of non-modified and nitrated α-synuclein during the incubations, the hydrodynamic radii of both forms of the protein at the early stages of fibrillation were estimated using dynamic light scattering (DLS). These DLS experiments were performed in parallel with the analysis of the reaction mixtures by SEC and the ThT assay. Initially the Stokes radius, R_S, of the non-modified protein was 32 ± 2 Å. This value is in good agreement with the results of previous studies [11,17,32,38], and corresponds to the monomeric natively unfolded α-synuclein. At early incubation times (well before the beginning of the fibril formation detected by the characteristic

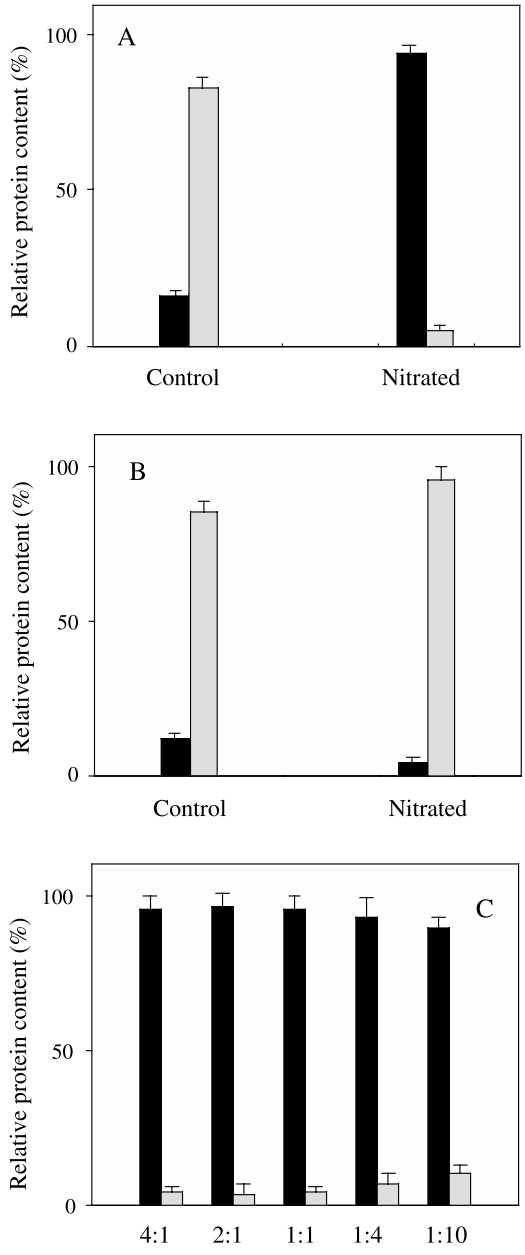

Fig. 2. α-Synuclein aggregation is inhibited by nitration. Incubated samples were centrifuged and the supernatant and pellet fractions analyzed for protein content using the Lowry assay. α-Synuclein (70 μM) was incubated in 20 mM buffer containing 100 mM NaCl at 37°C for 300 h. Panel A at pH 7.5, and panel B at pH 3.0. Panel C represents the results of inhibition studies on non-modified α-synuclein fibrillation in the presence of different concentrations of nitrated α-synuclein. The numbers show the molar ratios of the nitrated to non-modified protein. Black and gray bars represent the relative protein contents in the supernatant and pellet, respectively.

increase in the ThT fluorescence intensity) DLS showed that the majority of the non-modified protein was monomeric and characterized by a slightly increased R_S value (~35–37 Å) due to the presence of a small amount of dimer. This value of the Stokes radius represents a weighted average between monomer and dimer, due to technical limitations of the instrument. Corresponding SEC analyses also indicated the presence of a small amount of dimer. Furthermore, at these pre-fibrillation time points, DLS demonstrated that a small fraction of non-modified protein formed large oligomers, the size of which increased monotonically from ~200 to ~2000 Å (R_S), in accord with previous reports using different techniques [39]. Finally, almost no monomeric or small oligomeric species were detected when fibrils started to form, and the majority of protein in the soluble fraction of the reaction mixture possessed an R_S of >400 Å.

In contrast, even at time zero (i.e. at the beginning of the incubation) DLS showed that the majority of the nitrated α-synuclein (96%) formed small oligomers with R_S of 62 ± 3 Å, whereas the remainder of the protein was present as larger oligomers (R_S of 160 ± 10 Å). Parallel SEC/DLS analysis revealed that octamers represented the predominant species in the reaction mixture, but some dimers and tetramers were also present. The size and relative population of small oligomers did not change significantly during the incubation, whereas the small fraction of larger oligomers showed a time-dependent increase in R_S from 160 to 350 Å. The data thus indicate that nitrated α-synuclein has an increased propensity to form stable oligomers compared to normal, unmodified α-synuclein.

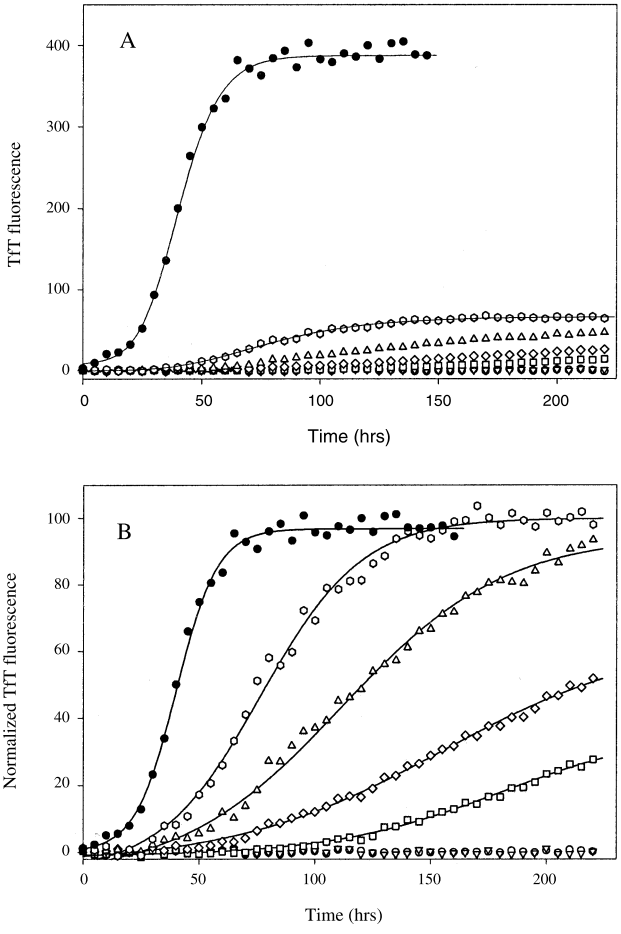

Fig. 3. Nitrated α-synuclein inhibits fibrillation of non-modified α-synuclein. A: Raw data. B: Normalized fluorescence intensity. The fibrillation kinetics were monitored by ThT fluorescence for 70 μM non-nitrated α-synuclein in 20 mM Na-phosphate buffer, 100 mM NaCl, pH 7.5 in the absence (black circles) or presence of 2.0 (inverted triangles), 1.0 (open squares), 0.50 (open diamonds), 0.25 (open triangles) and 0.10 (open hexagons) molar equivalents of the nitrated protein.

3.4. Nitrated α-synuclein inhibits fibrillation of the non-modified protein

Fig. 3A shows that the addition of nitrated α-synuclein to a solution of non-modified α-synuclein leads to very effective inhibition of fibrillation of the non-modified protein, and a significant decrease in the amount of ThT fluorescence was observed when the ratio of modified protein to non-modified α-synuclein was as low as 1:10. Fig. 3B shows that both lag-time and elongation rate are substantially affected by the addition of nitrated α-synuclein at sub-stoichiometric levels. The extent of these effects depends on the relative concentration of the modified protein, with larger concentrations showing stronger inhibitory effects. In fact, fibrillation was not observed (at least within the time-scale studied) when the ratio of nitrated to non-modified α-synuclein was 2:1 and higher. Analysis of the data presented in Fig. 3B revealed that the duration of the lag-time was 22.5 ± 0.5, 33.3 ± 0.6, 41.3 ± 0.6, 63.6 ± 0.7 and 106.7 ± 0.8 h, whereas the elongation rate was 0.113 ± 0.002, 0.0456 ± 0.0009, 0.0278 ± 0.0006, 0.0179 ± 0.0004 and 0.0082 ± 0.0002 h^{-1} when the ratio of nitrated to non-modified α-synuclein was 0, 1:1, 1:2, 1:4 and 1:10, respectively.

SEC analysis of incubation mixtures of nitrated and unmodified α-synuclein showed nitrotyrosine absorbance in the peak corresponding to the octamer (data not shown), indicating that these oligomers were hetero-oligomers. Fig. 2C shows that the amount of insoluble material decreases with increase in the relative concentration of the nitrated protein. Thus, nitrated α-synuclein effectively inhibited both fibrillation and amorphous aggregate formation from the non-modified protein.

3.5. Morphology of α-synuclein fibrils

Electron micrographs confirmed the lack of fibrils after incubation of either nitrated α-synuclein (Fig. 4B) or a 1:1 mixture with unmodified protein (Fig. 4C). The few fibrils observed from the nitrated α-synuclein were essentially indistinguishable from those of non-modified α-synuclein, Fig. 4A. Magnification of the image in Fig. 4C shows the presence of many spherical-shaped oligomeric intermediates of about 50–60 nm diameter (Fig. 4D).

4. Discussion

The data presented in this study unambiguously show that nitration of α-synuclein, in the absence of covalent dityrosine cross-linking, inhibits fibrillation in vitro. The mechanism of this inhibition involves formation of stable soluble oligomers, which are located off the fibrillation pathway. Perhaps more importantly, nitrated α-synuclein inhibits fibrillation of non-modified α-synuclein, even at sub-stoichiometric concentrations. In fact, effective inhibition of the fibrillation of non-modified protein occurred even when the nitrated α-synuclein accounted for only 10% of total protein. Notably, all three kinetic parameters characterizing the fibrillation assay, lag-time, elongation rate, and final intensity, showed strong dependence on the relative concentration of the nitrated protein. These results suggest that interaction between nitrated and non-modified forms of human α-synuclein leads to the formation of very stable hetero-oligomers, as seen in Fig. 4D. The formation of these non-fibrillogenic oligomers effectively decreases the concentration of fibrillation-prone non-modified

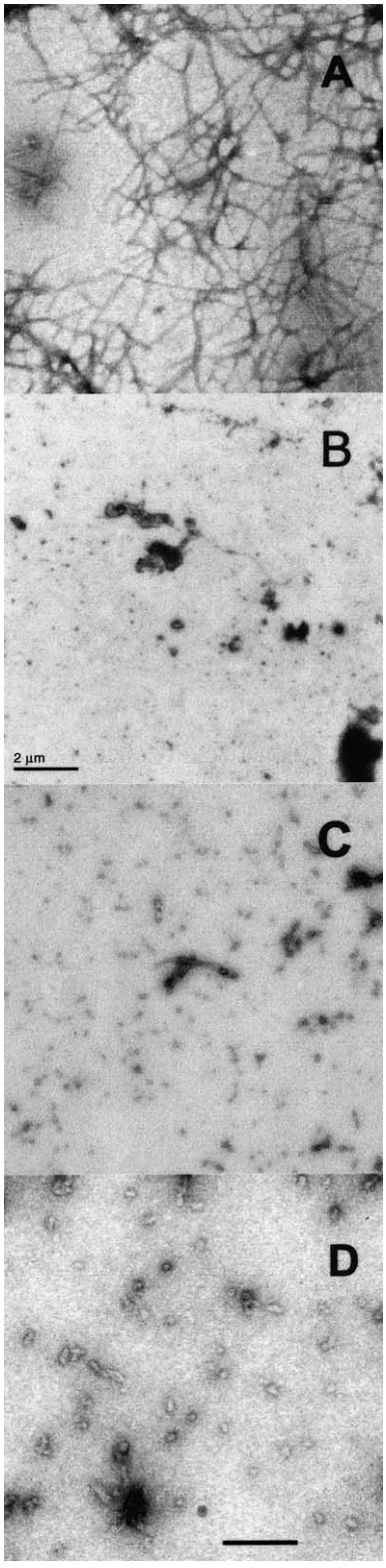

Fig. 4. Negatively stained transmission electron micrographs of α-synuclein fibrils prepared for the non-modified (A) and nitrated protein (B), as well as for the 1:1 mixture of modified and non-modified proteins (C). The scale bar indicates 2 μm in panels A–C. Panel D is a magnified image of panel C, showing spherical-shaped oligomeric intermediates (scale bar is 500 nm).

protein, and thus inhibits both the formation of nuclei and the growth of nascent fibrils. Fibrillation of recombinant α-synuclein has been shown to be strongly dependent on protein concentration [40]. The SEC data indicated that the molecular mass of the predominant oligomers of nitrated α-synuclein corresponded to an octamer. The fact that as little as 10% of nitrated α-synuclein inhibited fibrillation is consistent with the formation of hetero-oligomers consisting of approximately one nitrated to 10 unmodified α-synucleins.

We have previously shown that methionine-oxidized α-synuclein also suppressed fibrillation of α-synuclein. However, the mechanism of that inhibition was different, as oxidized protein affected primarily the duration of the lag-time, and did not affect the rate of elongation [37]. Similar inhibitory effects have been described for several pairs of homologous proteins. Examples include human β-/α- and γ-/α-synucleins [17]; mouse/wild type human and mouse/A53T human α-synucleins [41]; Aβ40/Aβ42 [42]; non-fibrillogenic γ-chain/fibrillogenic mutant β-chain of hemoglobin [43].

The stable oligomeric intermediates formed by, and in the presence of, nitrated α-synuclein, are similar to the species termed protofibrils by Lansbury and coworkers [39,44,45]. Since these species may form annular channels in membrane vesicles, they have been suggested as the potentially neurotoxic species in PD [44].

It is widely accepted that aggregation of α-synuclein is involved in the etiology of PD. It is also believed that oxidative stress may represent one of the key determinants in development of the disease [27–30,46]. In particular, it has been shown that extensive and widespread accumulation of nitrated α-synuclein is found in the Lewy bodies of PD and related synucleinopathies.

Based on these observations it has been concluded that the selective and specific nitration of α-synuclein in these disorders provides evidence to directly link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies [27]. However, our in vitro data on the effect of nitration of human α-synuclein on its fibrillation contradict these conclusions. In fact, we have shown that nitration effectively inhibits fibrillation of α-synuclein. Moreover, the addition of sub-stoichiometric concentrations of nitrated α-synuclein inhibits the fibrillation of non-modified α-synuclein due to the formation of non-productive hetero-oligomers. Our observations are consistent with the hypothesis that it is the formation of covalent intermolecular dityrosine cross-links, rather than nitration, which is responsible for α-synuclein fibrillation under oxidative conditions.

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