Formation and Removal of α-Synuclein Aggregates in Cells Exposed to Mitochondrial Inhibitors*

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Mitochondrial dysfunction has been associated with Parkinson’s disease. However, the role of mitochondrial defects in the formation of Lewy bodies, a pathological hallmark of Parkinson’s disease has not been addressed directly. In this report, we investigated the effects of inhibitors of the mitochondrial electron-transport chain on the aggregation of α-synuclein, a major protein component of Lewy bodies. Treatment with rotenone, an inhibitor of complex I, resulted in an increase of detergent-resistant α-synuclein aggregates and a reduction in ATP level. Another inhibitor of the electron-transport chain, oligomycin, also showed temporal correlation between the formation of aggregates and ATP reduction. Microscopic analyses showed a progressive evolution of small aggregates of α-synuclein to a large perinuclear inclusion body. The inclusions were co-stained with ubiquitin, 20 S proteasome, γ-tubulin, and vimentin. The perinuclear inclusion bodies, but not the small cytoplasmic aggregates, were thioflavin S-positive, suggesting the amyloid-like conformation. Interestingly, the aggregates disappeared when the cells were replenished with inhibitor-free medium. Disappearance of aggregates coincided with the recovery of mitochondrial metabolism and was partially inhibited by proteasome inhibitors. These results suggest that the formation of α-synuclein inclusions could be initiated by an impaired mitochondrial function and be reversed by restoring normal mitochondrial metabolism.

Many neurodegenerative diseases are associated with characteristic intracellular protein inclusions in the affected brain regions (1, 2). Lewy body (LB)† is one of the inclusions that is associated with Parkinson’s disease (PD) and related neurologic diseases, including dementia with Lewy bodies and Lewy body variant of Alzheimer’s disease (3). Aggregation of α-synuclein (α-syn) appears to be essential for the LB formation since fibrillar aggregates of α-syn are major components of LBs (4, 5), thus the diseases that are characterized by LBs are collectively referred to as α-synucleinopathies. α-Syn itself forms typical amyloids in solution by a nucleation-dependent mechanism (6–8). Importantly, two autosomal dominant mutations of the α-syn gene were linked to rare familial early-onset PD (9, 10), and the mutant proteins tend to aggregate more rapidly than the wild type (11–13). Transgenic flies modified to express human α-syn at various levels develop age-dependent pathological and behavioral changes that resemble human PD, including LB-like intraneuronal inclusions, loss of dopaminergic neurons, and a decline in locomotor activity (14). Also, transgenic mice that express human α-syn produced neuronal inclusions and dopaminergic presynaptic degeneration (15). In addition, the extent of formation of these LB-like inclusions has been shown to correlate with the expression level of α-syn in transgenic animals (15). These findings provide compelling evidence that the aggregation of α-syn is directly linked to LB formation, and therefore to the pathogenesis of α-synucleinopathies, especially PD. Although substantial progress has been made regarding structural and morphological changes during the fibrillation of isolated α-syn, physiological factors that influence the cellular aggregation process remain unknown.

Mitochondrial dysfunction has been implicated in pathogenesis of PD and other neurodegenerative diseases (16, 17). Several groups reported a systemic or local decrease of mitochondrial complex I activity (18–21) or immunoreactivity (22) in idiopathic PD. Another line of evidence for mitochondrial defects in PD came from studies where cybrids that contained mitochondria from PD patients showed reduced complex I activity (23, 24). In fact, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism is attributed to the inhibition of complex I by its metabolite, 1-methyl-4-phenylpyridinium (MPP⁺), which leads to reduction in mitochondrial ATP production (25, 26). Moreover, in a recent study administration of rotenone, an inhibitor of mitochondrial complex I, recapitulated the major pathological and behavioral features of PD, including selective loss of nigrostriatal dopaminergic neurons and α-syn-positive LB-like inclusions, indicating that the inhibition of complex I in the electron-transport chain (ETC) might be sufficient to trigger PD pathogenesis in a rat model (27). Although mitochondrial defects are clearly associated with PD, a direct role of mitochondrial dysfunction in LB formation has not been addressed. Here we provide evidence that the functional state of mitochondria might be a key determinant for aggregation of α-syn. In addition, the aggregates appeared to be eliminated by restoring normal mitochondrial metabolism, suggesting the presence of cellular mechanisms that actively disassemble and degrade protein aggregates.

MATERIALS AND METHODS

Construction of Recombinant Adenoviral Vector and Plasmids—Human wild type α-syn cDNA was amplified by polymerase chain reaction using the primer sets, 5’-ATCGATCTGGCCATGGATGTTATCTGAA-

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‡ The abbreviations used are: LB, Lewy body; PD, Parkinson’s disease; ETC, electron transport chain; syn, synuclein; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Ub-Pr, ubiquitin-proteasome.
AGGA and 5'-ATGGATGTCTTCAAGAAGG and 5'-412 et al. (vitrogen) according to Lee (29). Antibodies used were LB509 (Zymed Laboratories, Inc., South San Francisco, CA) for ubiquitin antibody (Chemicon International, Inc., Temecula, CA) or oligomycin (0.1 µg/ml in Me2SO; Sigma) or thioflavin S (Sigma) for 8 min and washed three times with 80% ethanol for 5 min each before the antibody incubations (30). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was carried out using the kit from Roche Molecular Biochemicals (Mannheim, Germany). ATP was assayed using a recombinant adenoviral vector system, which yielded homogeneous expression in nearly 100% of the cells. Cells were treated with two ETC inhibitors, rotenone, a complex I (NADH-coenzyme Q reductase) inhibitor, and oligomycin, a complex V (F$_F$-ATP synthase) inhibitor. At each time point, cell extracts were fractionated to the Triton X-100-soluble and -insoluble fractions and analyzed by Western blot analysis. After 48 h of incubation in the presence of rotenone, the cells produced visible amounts of SDS-resistant α-syn aggregates, and by 72 h, most of the α-syn was in aggregated form (Fig. 1A). Vehicle alone had almost no effect on α-syn aggregation during the time course we examined (Fig. 1C). Rotenone did not influence the aggregation of α-syn in cell-free assays, suggesting that the effect of rotenone was not targeted directly to the protein itself (Fig. 1D). Oligomycin, although affecting a different target in the ETC, induced the aggregation of α-syn with a similar time course, supporting the idea that the ETC inhibition leads to the aggregation of α-syn (Fig. 1B).

There are other proteins, such as β-syn, γ-syn, and synoretin, that are closely related to α-syn (31, 32). Despite the sequence homology within the synuclein family of proteins, only α-syn is found in LBs (33). Furthermore, in vitro studies showed that α-syn is much more prone to aggregate than other syns and does not cross-seed β- or γ-syn (34, 35). To compare the effects of ETC inhibition on α-, β-, and γ-syns, cells that transiently expressed Myc/His-tagged synucleins were treated with rotenone, and the aggregation was monitored in the Triton-insoluble fractions. The antibody against Myc-tag was used to quantify the amounts of monomer and aggregates of each protein. Consistent with the previous in vitro studies with purified recombinant proteins (34, 35), only α-syn produced aggregates at day 2 despite the fact that the expression level of β-syn was much higher than that of α-syn. This data suggests that α-syn has highest aggregation tendency in the syn family in response to altered mitochondrial metabolism (Fig. 2).

In PD and other related synucleinopathies, the aggregation of α-syn is often manifested by the presence of large cytoplasmic inclusion bodies called LBs (3). We, therefore, investigated the formation of inclusion bodies in rotenone-treated cells microscopically (Fig. 3). Before rotenone treatment (t = 0 in Fig. 3A), α-syn was diffusely distributed throughout the entire cytoplasm. Dispersed staining of small aggregates began to appear at 24 h and increased with time. A small fraction of the cells with α-syn aggregates started to develop large inclusions in the perinuclear region by 48 h and the population increased with time. At 48 and 72 h, about 10% of cells with aggregated α-syn had large perinuclear inclusions.

Immunohistochemical studies showed that LBs contain not only α-syn, but also ubiquitin and subunits of proteasome (33, 36). To determine the presence of these proteins in the inclusion, we co-stained the rotenone-treated cells with antibodies against α-syn and ubiquitin or the α-subunit of 20 S proteasome. Both anti-ubiquitin and anti-α-subunit antibodies co-
stained with α-syn antibody in the perinuclear inclusions (Fig. 4, A and B). Thus, the perinuclear inclusions that were α-syn-positive contained ubiquitin and the proteasome subunit. In contrast, small aggregates that were dispersed in the cytoplasm contained neither ubiquitin nor the proteasome subunit (Fig. 4). We have not detected any small α-syn aggregates that were positive for either of these proteins.

Ubiquitin was also detected in the SDS-resistant, high molecular weight protein complex by Western blotting (data not shown). However, whether α-syn was directly ubiquitinated is not clear because of several reasons. First, α-syn aggregates and ubiquitin-positive aggregates can be separate entities, which happen to run at a similar rate on the SDS gel. Co-immunostaining of the perinuclear inclusions often showed segregation of these proteins (Fig. 4A). Second, α-syn may not be ubiquitinated directly, but simply co-aggregates with other ubiquitinated proteins. Analysis of monomeric proteins dissociated from aggregates will provide the direct answer to this problem.

Recently, the formation of perinuclear inclusions called aggresomes were described as a general response of cells to an accumulation of misfolded proteins (37). These aggresomes contain γ-tubulin, since they form near the microtubule organizing center, and are often surrounded by vimentin filaments (37).
case for typical aggresome-containing cells (37).

α-Syn aggregates in LBs are known to have a structure similar to amyloids, which are characterized by an ordered array of crossed β-sheet structure (6, 7). This structure can be selectively recognized by thiazole dyes, such as thioflavin T and S, good indicators of amyloid-like conformation (38, 39). To obtain conformational information, we double-stained rotenone-treated cells with an anti-α-syn antibody and thioflavin S. As described above, two forms of aggregates can be detected after rotenone treatment, small cytoplasmic aggregates and large perinuclear inclusions. The perinuclear inclusions in rotenone-treated cells were strongly stained with thioflavin S, while the small aggregates were thioflavin S-negative (Fig. 5).

To evaluate the functional defects of mitochondria in the cells exposed to the ETC inhibitors, we measured ATP levels as a marker of mitochondrial metabolism after rotenone or oligomycin treatment. When the cells were treated with rotenone, the reduction in ATP preceded and paralleled the aggregation of α-syn up to 72 h, with reductions of 23, 87, and 94% at 24, 48, and 72 h, respectively (Fig. 6A). Oligomycin treatment resulted in a similar time course of ATP reduction, which again paralleled the aggregation of α-syn (Fig. 6B). These data suggest that functional defects in the ETC may cause the aggregation of α-syn. To test whether mitochondria can recover after removal of the ETC inhibitors, the cells were cultured with rotenone or oligomycin for 48 h, then the medium was replaced with the inhibitor-free medium (Fig. 7A). ATP levels were slowly restored in the wash-out phase. At 48 h of wash-out, the rotenone-treated cells had almost fully recovered from the ATP deficit while the oligomycin-treated cells had recovered by 50%, suggesting a functional recovery of mitochondria (Fig. 6, C and D).

To determine whether the aggregation of α-syn can be reversed by the removal of the ETC inhibitors, the extent of SDS-resistant α-syn aggregates were investigated in the wash-out experiment (Fig. 7A). During this period, the α-syn aggregates gradually diminished, and by 48 h of wash-out, most of the aggregates had disappeared (Fig. 7, B and C). Disappearance of the aggregates paralleled the recovery of mitochondrial metabolism, which was monitored by the cellular ATP level. Even after longer aggregation period, which produced more α-syn aggregates, replacement with rotenone-free medium resulted in a progressive removal of the aggregates (Fig. 7D). This phenomenon could be due to the death of the cells with α-syn aggregates. However, TUNEL staining showed that the number of dying cells was minimal (4–8% of total cells), with no correlation between α-syn aggregation and apoptotic cell death during the entire time course of the experiment (data not shown). An independent assessment of cell death by nuclear morphology produced similar results (data not shown). Therefore, the disappearance of the α-syn aggregates is not due to the

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selective loss of the cells with the aggregates. Rather, there may be an active mechanism for the removal of the pre-existing aggregates in the cells. Disappearance of aggregates during the wash-out phase was also analyzed microscopically (Fig. 7E). Consistent with the Western analysis, replacement with rotenone-free medium resulted in a disappearance of small dispersed aggregates. However, the number of cells with the large perinuclear inclusions (about 8% of total cells) remained throughout the wash-out phase up to 2 days, suggesting that these large inclusions are more resistant to the aggregate-cleanup mechanism than the small aggregates.

Disappearance of the aggregates in the wash-out phase was not accompanied by a concomitant increase of monomeric α-syn. This suggests an elimination of aggregates by degradation rather than by simple disassembly. To investigate the role of the proteasome system in the degradation of α-syn aggregates, we studied the effects of proteasome inhibitors on the disappearance of the aggregates during the rotenone wash-out.

Fig. 8. Inhibition of the aggregate-removal by proteasome inhibitors. COS-7 cells overexpressing α-syn were treated with rotenone for 56 h (lane 1), then cultured in a rotenone-free medium containing Me2SO (lane 3), MG132 (5 μM, lane 4), ALLN (5 μM, lane 5), or lactacystin (20 μM, lane 6) for 16 h. Lane 2 shows the aggregates formed during 72-h rotenone treatment without the wash-out phase. Triton-insoluble fractions were analyzed by Western blotting using LB509 antibody.

Treatment of cells with rotenone for 56 h resulted in high molecular weight aggregates. While continued incubation for another 16 h in the presence of rotenone produced more aggregates, a parallel incubation in the absence of rotenone resulted in a disappearance of pre-existing aggregates (Fig. 8). However, addition of proteasome inhibitors MG132, ALLN, or lactacystin in the wash-out medium partially blocked the disappearance of the aggregates, suggesting that the proteasome system might be at least partly responsible for the degradation of α-syn aggregates.

DISCUSSION

Our study provides the first cell-based evidence that mitochondrial dysfunction may result in α-syn aggregation. One of the outcomes of ETC inhibition is an increased production of free radicals, hence increased oxidative stress (17). In recent studies, several groups have shown that the aggregation of α-syn could be promoted by oxidative and nitratative stresses (30, 40, 41). Indeed, oxidizing and nitrating agents induced dityrosine cross-linking of recombinant α-syn and stabilized pre-assembled aggregates (42). Furthermore, accumulation of nitrated α-syn was demonstrated in the inclusions of PD, dementia with Lewy bodies, Lewy body variant of Alzheimer’s disease, and multiple system atrophy, implicating the role of oxidative stress in LB formation in α-synucleinopathies (43). Another outcome of mitochondrial dysfunction is a defect in energy production (ATP generation). In our study, we showed tight temporal correlations between ATP level and α-syn aggregation both in depletion and recovery phases, suggesting that an impaired energy supply may also play a role in α-syn aggregation.

Protein aggregation is considered to be a manifestation of a disturbed cellular protein-folding homeostasis, which is maintained by at least two defense mechanisms against damaged (misfolded) proteins: degradation by the ubiquitin-proteasome (Ub-Pr) system and the chaperone-mediated refolding system (44). Impairment of these systems, most of which are dependent on ATP, will cause accumulation of the misfolded proteins. Therefore, while oxidative stress can increase the rate of protein misfolding, the concomitant reduction in ATP levels can decrease the rate at which the cells rescue or remove the misfolded proteins, thus resulting in protein aggregates. Interestingly, unlike globular proteins, α-syn in isolation does not appear to have any stable structure (45). To initiate the aggreg-
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gation process, α-syn has to undergo a structural transition to form an aggregation-prone, partially folded intermediate, which is equivalent to the misfolded proteins in the aggregation process of globular proteins. In fact, the presence of the partially folded intermediate and the stabilization of this conformation in the α-syn aggregation process were demonstrated in recent studies (46, 47). Furthermore, the degradation of α-syn is mediated by the ubiquitin-proteasome system (48, 49). These results suggest that the aggregation of α-syn might be under control of the same defense mechanism as globular proteins. Therefore, the correlation between the ATP level and α-syn aggregation shown in our study suggests that the reduction in energy production, hence the impaired defense mechanism against misfolded proteins, is likely to contribute to the aggregation of α-syn. During the rotenone treatment, chymotrypsin-like activity of proteasome in the cell lysates was measured using a fluorogenic peptide substrate. In this assay, the proteasome activity was only slightly decreased by about 10% in the rotenone-treated cells compared with the vehicle-treated cells.² It is unlikely that this slight decrease in catalytic activity of 20 S proteasome core is responsible for the protein aggregation, given that this activity is quite resistant to ATP-depletion when peptidic substrate is used (50, 51). Rather, other ATP-dependent processes in Ub-Pr system, such as ubiquitination cascade and regulatory activity of 19 S proteasome, might be impaired. Further characterization of these processes should unveil the role of energy metabolism and Ub-Pr system in α-syn aggregation.

Recently, a mechanism that explains the formation of protein inclusions in eukaryotic cells was proposed through the description of a structure called an aggresome. Aggresomes are formed near the microtubule organizing center by accumulation of small aggregates that are initially dispersed in the cytoplasm and delivered to the perinuclear region by dynein-dependent retrograde transport on microtubules (37, 52). In the present study, we showed that the α-syn-positive inclusions share some properties with the aggresomes. First, the temporal changes in the size and the distribution of α-syn aggregates, from small dispersed aggregates to large perinuclear inclusions, resemble those in aggresome formation. Second, the α-syn-positive inclusions contain γ-tubulin, a marker of the microtubule organizing center. And finally, the inclusions are surrounded by what appear to be vimentin filaments, reminiscent of the “vimentin cages” that surround the aggresomes. These aggresome-like properties were also found in other inclusions that are relevant to neurodegenerative diseases, such as the inclusions of mutant Cu,Zn-superoxide dismutase and mutant huntingtin fragment (53, 54).

Although small aggregates dispersed in the cytoplasm appeared to be precursors for the large perinuclear inclusions, thioflavin S staining showed that conformations of α-syn in these structures might be different: small aggregates were thioflavin S-negative, whereas large inclusions were thioflavin S-positive. This interesting distinction between small aggregates and large perinuclear inclusions suggests that the structural transition of α-syn into highly ordered β-sheet-rich conformation occurred after the aggregates accumulated in the perinuclear region. This thioflavin S reactivity can be explained by the “nucleated conformational conversion” model proposed by Serio et al. (55) in their work on the fibrillation of the N-terminal and middle regions of Sup35 (so called NMF fragment), a yeast prion. In this model, elongation of crossed β-sheet amyloid is mediated by templated conformational conversion of less structured oligomeric intermediate. Less

² H.-J. Lee and S.-J. Lee, unpublished observation.

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