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Oxidative Stress Involvement in α-Synuclein Oligomerization in Parkinson's Disease Cybrids

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Abstract

Mitochondrial dysfunction, oxidative stress, and α-synuclein oligomerization occur in Parkinson disease (PD). We used an *in vitro* PD cybrid approach that models these three phenomena specifically to evaluate the impact of mitochondria-derived oxidative stress on α-synuclein oligomerization. Compared with control cybrid cell lines, reactive oxygen species (ROS) production and protein oxidative stress markers were elevated in PD cybrids. The antioxidants CoQ_{10} and GSH attenuated changes in PD cybrid peroxide, protein carbonyl, and protein sulfhydryl levels. Elevated PD cybrid α-synuclein oligomer levels were also attenuated by CoQ_{10} and GSH. In PD cybrids, α-synuclein oligomerization was activated *via* a complex I–mediated increase in the free tubulin/polymerized tubulin ratio. CoQ_{10} but not GSH increased complex I activity, restored ATP to control levels, and normalized the PD cybrid free tubulin/polymerized tubulin ratio. Overall, we conclude that two different antioxidants can decrease α-synuclein oligomerization whether by improving mitochondrial function or by preventing protein carbonylation or both. We conclude that mitochondrial dysfunction can induce α-synuclein oligomerization *via* ATP depletion–driven microtubule depolymerization and *via* ROS increase–driven protein oxidation. *Antioxid. Redox Signal*. 11, 439–448.

Introduction

Parkinson's disease (PD) is characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta, and by the formation of Lewy bodies (LB), which are intracytoplasmic inclusions composed mainly of α-synuclein, ubiquitin, and tubulin (17). Moreover, mitochondrial complex I deficiency was identified in both degenerating and nondegenerating PD tissues (reviewed in 6). To address the relevance of mitochondrial function in PD, the cytoplasmic hybrid (cybrid) technique, previously described by Esteves and colleagues (15), has been applied. It was described in PD cybrids a stable decrease in complex I activity, increased reactive oxygen species (ROS) production, decreased ATP levels, and increased susceptibility to MPP^+ (8, 15, 40). Moreover, the generation of fibrillar and vesicular inclusions was reported in cybrid models of sporadic PD that replicate the essential antigenic and structural features of LB, without the need for exogenous protein expression or inhibition of mitochondrial or proteasomal function (14, 42).

As oxygen radicals are a by-product of mitochondrial respiration, and mitochondrial dysfunction can cause an increase in ROS, these organelles may contribute to an overall increase in cellular oxidative damage. Substantial data from postmortem studies show increased oxidative damage in PD. A consistent increase in lipid peroxidation, an increase in protein carbonyl groups, and an increase in 8-hydroxy-2-deoxyguanosine concentration in DNA have been reported in PD substantia nigra (10, 16). In addition, reduced glutathione (GSH), the major antioxidant within cells that acts as free radical scavenger and helps to regulate the thiol disulfide concentration of different proteins (27, 46), is markedly decreased in the substantia nigra of PD patients (35). Furthermore, it was described that the level of coenzyme Q (CoQ), an essential cofactor of the electron-transport chain (accepts electrons from complexes I and II) that possesses antioxidant properties, was signif-

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icantly lower in mitochondria from PD patients as compared with that in age-matched controls (36).

Oxidation is associated with α-synuclein aggregation and has been proposed as one of the mechanisms responsible for the *in vitro* formation of cross-linked α-synuclein oligomers (12, 37). Regarding the role of oxidative stress in α-synuclein aggregation, Takahashi *et al*. (41), in recent experiments, showed that exposure of $FeCl_2$ to a neuronal cellular model led to the formation of α-synuclein inclusions and oligomers, indicating that oxidative stress can contribute to protein aggregation.

Microtubules are currently being proposed to have a role in PD pathogenesis. Cappeleti and co-workers (5) demonstrated that MPP^+ specifically affects microtubule dynamics *in vitro*. Additionally, Ren and Feng (32) showed that rotenone, also a complex I inhibitor, induced microtubule depolymerization in primary midbrain neuronal cultures. In addition, it has been proposed that tubulin can stimulate α-synuclein fibrillization in yeast and in cell-free systems (1, 24). We have reported that a decrease in ATP levels, due to complex I inhibition, induced an increase in free tubulin that led to α-synuclein oligomerization (14). We now report that mitochondria-dependent ROS formation also contributes to α-synuclein oligomerization and microtubule depolymerization. Our results support the view that a primary mitochondrial respiratory-chain defect is responsible for α-synuclein oligomerization due to an increase in ROS production and to ATP depletion.

Material and Methods

Subject participation was approved through the Institutional Review Board of the University Hospital of Coimbra. PD subjects were evaluated at the Neurological Unit of the University Hospital of Coimbra and met the Gelb and coworkers criteria (18) for probable PD. Individuals in the PD group did not have indications of any other neurodegenerative diseases. Healthy individuals were used as controls and were cognitively normal and age matched to PD subjects. The mean age of the CT group ($n = 3$) was 64.3 ± 8.4 years, and for the PD group ($n = 2$) was 65.0 ± 5 years.

Chemicals and cell media

CoQ_{10} was obtained from Fluka, and GSH and H_2O_2 were obtained from Sigma (St. Louis, MO). Optimem medium was obtained from Gibco-Invitrogen (Eugene, OR). Nondialyzed and dialyzed fetal bovine serum (FBS) was obtained from Gibco-Invitrogen. NT2 ρ0 cell growth medium consisted of Optimem supplemented with 10% nondialyzed FBS, 200 μg/ml sodium pyruvate, 150 μg/ml uridine, and 100 IU/ml penicillin with 50 μg/ml streptomycin (pen-strep). NT2 cybrid selection medium consisted of Optimem supplemented with 10% nondialyzed FBS and pen-strep. Cybrid expansion medium consisted of Optimem supplemented with 10% dialyzed FBS and pen-strep.

Platelet mitochondria preparation

After informed consent, 10 ml of blood was collected through venipuncture in tubes containing acid-citrate-dextrose as an anticoagulant. Mitochondria were obtained from human platelets according to previously described methods (25). Platelet mitochondria protein concentrations were measured with the Bradford protein assay, in which bovine serum albumin was used as the standard (4).

Cybrid cell culture

To create the cybrid cell lines for this study, we used a teratocarcinoma cell line lacking mtDNA (NT2-Rho0 cell line) that does not possess a functional ETC and is autotrophic for pyruvate and uridine (39). In brief, NT2-Rho0 cells were repopulated with platelet mitochondria from either healthy individuals or PD patients (CT cybrids and PD cybrids, respectively). Seven days after fusion, cybrid cells were placed in selection medium containing 10% dialyzed fetal calf serum and lacking pyruvate and uridine, as previously described (7, 39). These conditions result in the selective death of Rho0 cells that have not been repopulated with donor mitochondria.

After selection was complete, cybrid cells were maintained in culture medium (Dulbecco's modified Eagle's medium, DMEM, supplemented with 10% bovine calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin) in a humidified 95% air/5% CO_2 incubator at 37°C.

For CoQ_{10}, GSH, or H_2O_2 supplementation experiments, cybrid cell lines were placed in media containing 0.15 μ*M* CoQ_{10}, 2 m*M* GSH, or 100 μ*M* H_2O_2, respectively. Flasks were maintained in these media at 37°C, 5% CO_2 for 24 h before harvesting.

Fluorimetric reactive oxygen species production

ROS production was measured by using a nonfluorescent probe 2,7′-dichlorofluorescein-diacetate ($DCFH_2$-DA). $DCFH_2$-DA readily crosses the cell membranes, acetates being cleaved by intracellular esterases to form 2,7′-dichlorohydrofluorescein (DCF H2), which was kept trapped in the cell. In the presence of ROS, DCF H_2 is oxidized to DCF (dichlorofluorescein), a highly fluorescent compound.

After a 24-h treatment, cells were washed once with PBS (at 37°C), and then incubated with 20 μ*M* $DCFH_2$-DA (from a stock solution of 1 m*M* in DMSO) in Krebs medium, at 37°C, and put in the incubator in light-protected conditions, for 30 min. The incubation medium was then removed, and after a single wash with Krebs medium, cells were left with Krebs medium.

Fluorescence was monitored every 1 min for 1 h, at 37°C, by using a Spectramax GEMINI EM fluorocytometer (Molecular Devices), with excitation and emission wavelengths corresponding to 480 and 550 nm, respectively, with cut-off at 530 nm.

Determination of protein sulfhydryl groups

Sulfhydryl groups were determined in CT and in PD cybrids in the presence or absence of antioxidants, by using DTNB [5′,5′-dithio-bis(2-nitrobenzoic acid)], according to the method of Di Monte *et al*. (11).

Cellular proteins were precipitated with 10% TCA (trichloroacetic acid) and then incubated with 100 m*M* DTNB in 0.5 *M* Tris-HCl (or 0.5 *M* Tris-HCl alone for the blanks), in the dark, for 20 min at room temperature. The thiol groups were determined spectrophotometrically at 412 nm. Data were expressed as nanomol SH per milligram protein, calculated on the basis of a reduced glutathione calibration curve.

Determination of protein carbonyl groups

Protein carbonyl content was determined as described by Levine *et al*. (26), with slight modifications in CT and PD cybrids cellular extracts in the presence or absence of antioxidants. Cellular proteins were precipitated with 10% TCA (trichloroacetic acid) and then incubated with 10 m*M* DNPH (dinitrophenylhydrazine) in 2 *M* HCl (or 2 *M* HCl alone for the blanks), for 1 h at room temperature. The protein hydrazone derivatives were precipitated with 20% trichloroacetic acid, and the precipitates were washed 3 times with 1 ml ethanol/ethylacetate (1:1). During each washing, the homogenized pellet was vortexed and left in the washing solution for 10 min at room temperature before centrifugation. The final pellet was resuspended in 6 *M* guanidine HCl, and incubated for 15 min at 37°C. The carbonyl content was determined spectrophotometrically, by using a microplate reader, at 360 nm on the basis of molar absorbance coefficient of 22,000 M/cm.

Immunoprecipitation

Cells were scraped in buffer containing 20 m*M* Tris, 100 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA (pH 7.0), and protease inhibitors (200 m*M* PMSF and a commercial protease inhibitor cocktail). CT and PD cybrids cell suspensions were centrifuged at 20.000 *g* for 10 min at 4°C. Supernatants were removed and stored at −80°C. The protein concentration of each sample was determined by the Bradford method (4): 500 μg of cell lysates was incubated with primary antibody (1:50 α-synuclein antibody, Biotechnology, Inc., Santa Cruz, CA), overnight at 4°C, and with gentle agitation. Lysates were then incubated with 100 μl of protein-A beads for 2 h at 4°C and with gentle agitation. After completing this incubation, lysate tubes were centrifuged at 65 *g* for 5 min at 4°C, the supernatant was removed, and the beads were washed in the previously described buffer 7 times (each time centrifuging at 4°C and removing the supernatant). For the two first washes, the buffer was supplemented with 1% Triton X-100. For the next three washes, the buffer was supplemented with 1% Triton X-100 and 500 m*M* NaCl. The two final washes were performed with unsupplemented buffer. Finally, the last supernatant was removed, and 25 μl of 2× sample buffer was added. The sample was boiled at 95–100°C for 5 min to denature the protein and to separate it from the protein-A/G beads. The boiled proteins were centrifuged at 20,000 *g* for 5 min at room temperature, and the supernatants were collected. The resulting immunoprecipitated proteins were derivatized with DNPH.

Immunoblotting

Cells were scraped in a buffer containing 25 m*M* HEPES, 1 m*M* EDTA, 1 m*M* EGTA, 2 m*M* $MgCl_2$, and protease inhibitors (0.1 *M* PMSF, 0.2 *M* DTT, 1% Triton X-100 and 1:1,000 dilution of a protease-inhibitor cocktail). Cells were then frozen 3 times on liquid nitrogen and centrifuged at 20,000 *g* for 10 min. The supernatants were removed and stored at −80°C. Protein concentrations were determined by the Bradford method (4), and equal amounts of protein (30 or 60 μg) were used in each immunoblotting evaluation. For the SDS-PAGE experiments, samples were resolved by electrophoresis in SDS-polyacrylamide gels and transferred to PVDF membranes. For the PAGE experiments, samples were not boiled and were diluted 1:2 in a specific sample buffer. Nonspecific binding was blocked by gently agitating the membranes in 5% nonfat milk and 0.1% Tween in TBS for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4°C with gentle agitation (1:500 α-synuclein, Biotechnology, Inc.; 1:10,000 α-tubulin, Sigma). Blots were washed with TBS containing 0.1% nonfat milk and 0.1% Tween 3 times (each time for 15 min) and then incubated with the secondary antibodies (1:20,000, anti-mouse) for 2 h at room temperature with gentle agitation. After three washes, specific bands of interest were detected by developing with ECF-detection reagent. Fluorescence signals were detected by using a Biorad Versa-Doc Imager.

Immunocytochemistry and thioflavin S staining

PD and CT cybrid cell lines were grown on coverslips in 12-well plates. Cells were washed twice with PBS and fixed for 30 min at room temperature with 4% paraformaldehyde. The fixed cells were washed again with PBS, permeabilized with 0.2% Triton X-100, and blocked with 3%. BSA Then cells were incubated with primary antibody (1:400 α-synuclein, Biotechnology, Inc.) for 1 h and subsequently with secondary antibody (1:250 alexa fluor 594; Molecular Probes, Eugene, OR). Cells were visualized under a confocal microscope. To evaluate β-sheet protein aggregates, cells incubated with α-synuclein antibody were incubated for 20 min with 0.5% thioflavin S (Sigma), in the dark at room temperature, and then washed quickly in 70% ethanol. After this, cells were washed 3 times with PBS, and visualized by using a confocal microscope.

NADH-ubiquinone oxidoreductase assay

Complex I activity was determined by a modified version of Ragan *et al*. (31), which follows the decrease in NADH ab-

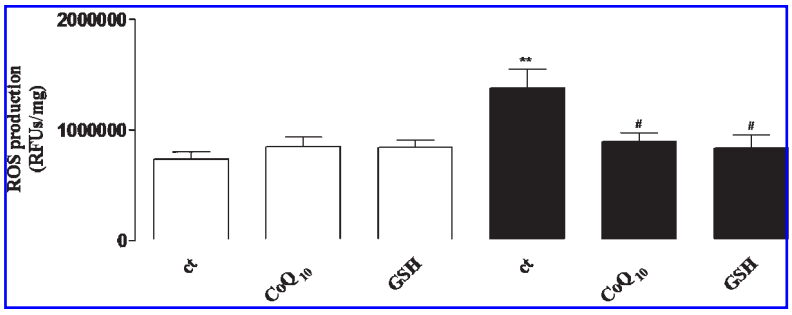

FIG. 1. Free radical production in PD cybrids. The increase in peroxide level production in PD cybrids cytosol, determined by using $DCFH_2$-DA, was prevented by CoQ_{10} and GSH treatment. Data are expressed as RFU per mg of protein, with the mean ± SEM from five different experiments. $^{**}p < 0.01$, significantly different when compared with untreated CT cybrids. $\#p < 0.05$, significantly different when compared with untreated PD cybrids. *White bars*, CT cybrids; *black bars*, PD cybrids.

sorbance at 340 nm that occurs when ubiquinone (CoQ_1) is reduced to form ubiquinol. The reaction was initiated by adding CoQ_1 (50 μM) to the 30°C reaction mixture. After 5 min, rotenone (10 μM) was added, and the reaction was monitored for another 5 min. Complex I activity was expressed both as nanomoles per minute per milligram of protein, and as the ratio of complex I activity to citrate synthase activity.

Analysis of adenosine nucleotides

After the 24-h cell plating–incubation period, medium was removed. Cells were extracted, on ice, with 0.4 *M* perchloric acid. Cells were next centrifuged at 14,000 rpm for 5 min at 4°C. The resulting pellets were solubilized with 1 *M* NaOH to analyze protein content further with the Bradford method (4). The resulting supernatants were neutralized with 5 *M* KOH and 2.5 *M* Tris, pH7-8, and then centrifuged at 14,000 rpm for 5 min at 4°C. These supernatants were assayed for adenine nucleotides (ATP, ADP, and AMP) by separation in a reversed-phase HPLC as described by Stocchi and colleagues (38).

Preparation of cell extracts containing soluble and polymeric tubulin

To prepare the soluble and polymeric fractions of cell tubulin, we washed the twice very gently with a microtubule stabilizing buffer (0.1 *M N*-morpholinoethanesulfonic acid, pH 6.75, 1 m*M* MgSO4, 2 m*M* EGTA, 0.1 m*M* EDTA, and 4 *M* glycerol). Soluble proteins were extracted at 37°C for 4 to 6 min in 100 μl of microtubule stabilizing buffer containing 0.1% Triton X-100. The remaining fraction in the culture dish was scraped in 100 μl of 25 m*M* Tris (pH 6.8), 0.5% SDS, and frozen 3 times in liquid nitrogen. Protein was quantified by the Bradford method (4). Samples were subjected to SDS-PAGE by using α-tubulin antibody (1:10,000, Sigma).

Data acquisition and analysis

All data result from the analysis of duplicates per experimental condition in at least three independent experiments and are expressed as the mean ± SEM. The *p* values were calculated with one-way ANOVA followed by a *post hoc* Dunnett's or Bonferroni's *t* test, as appropriate.

Results

Oxidative stress increase in PD cybrids

Inefficient electron transport through the mitochondrial respiratory complexes can lead to reduced ATP synthesis and the generation of ROS as a by-product. An increased level of ROS can lead to damage of macromolecules within the cell, such as lipids, proteins, and DNA. Therefore, oxidative damage to macromolecules may alter their function and thereby lead to impaired cellular functioning and subsequently to cell death.

PD cybrids with inherent mitochondrial dysfunction showed increased levels of ROS as compared with CT cybrids (Fig. 1). To try to reverse this increased production of ROS, we treated cells with two different, previously described, antioxidants, CoQ_{10} and GSH. We observed that both significantly decreased ROS production in PD cybrids to levels similar to untreated CT cybrids (Fig. 1).

Next we evaluated markers of protein oxidation, including changes in the content of protein carbonyls and protein sulfhydryl groups. In many cases, reactive carbonyls are produced as a consequence of oxidative stress. Additionally, protein thiols, which are essential for maintaining enzyme activities, are susceptible targets for a wide range of proox-

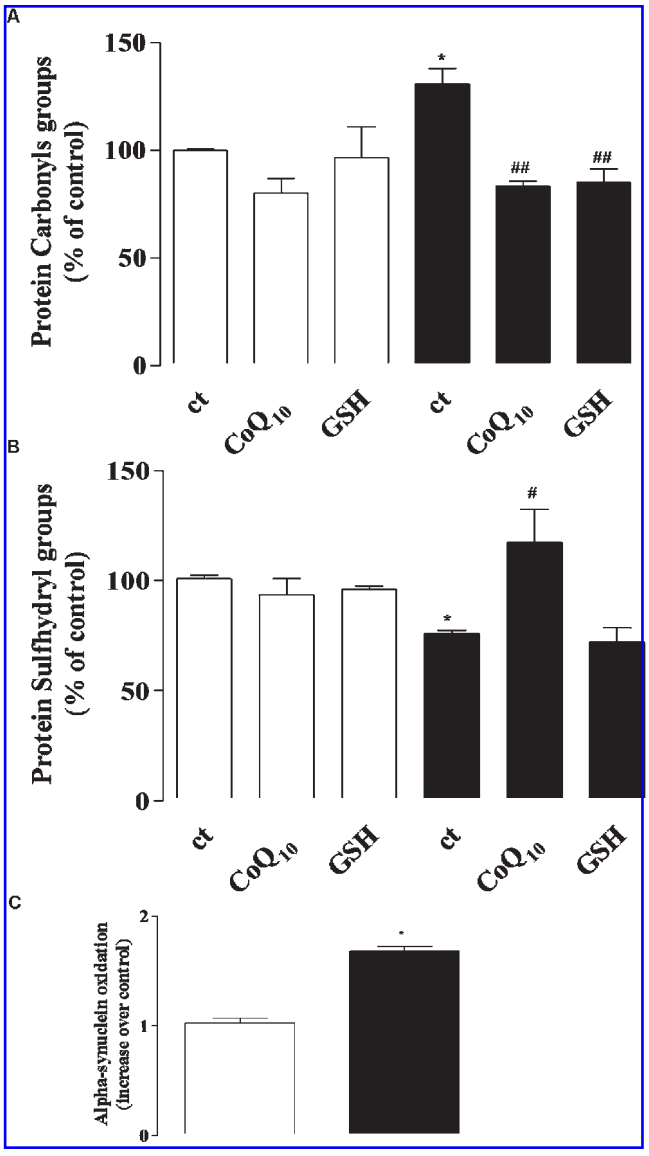

FIG. 2. Protein oxidation in PD cybrids. (**A**) Protein carbonyl groups increase in PD cybrids was prevented by CoQ_{10} and GSH treatments. Carbonyl groups were determined spectrophotometrically, by using DNPH. Results are expressed as a percentage of control, with the mean ± SEM from four independent experiments. (**B**) Protein sulfhydryl groups decrease in PD cybrids was prevented by CoQ_{10}. GSH did not prevent the decrease in protein SH. Sulfhydryl groups were determined spectrophotometrically, by using DTNB. Data are expressed as a percentage of control, with the mean ± SEM from four different experiments. (**C**) α-Synuclein oxidation was determined spectrophotometrically, by using DNPH, after immunoprecipitation. Data are expressed as a percentage of control, with the mean ± SEM from two different experiments. $^*p < 0.05$, significantly different as compared with untreated CT cybrids. $\#p < 0.05$, $\#\#p < 0.01$ significantly different as compared with untreated PD cybrids. *White bars*, CT cybrids; *black bars*, PD cybrids.

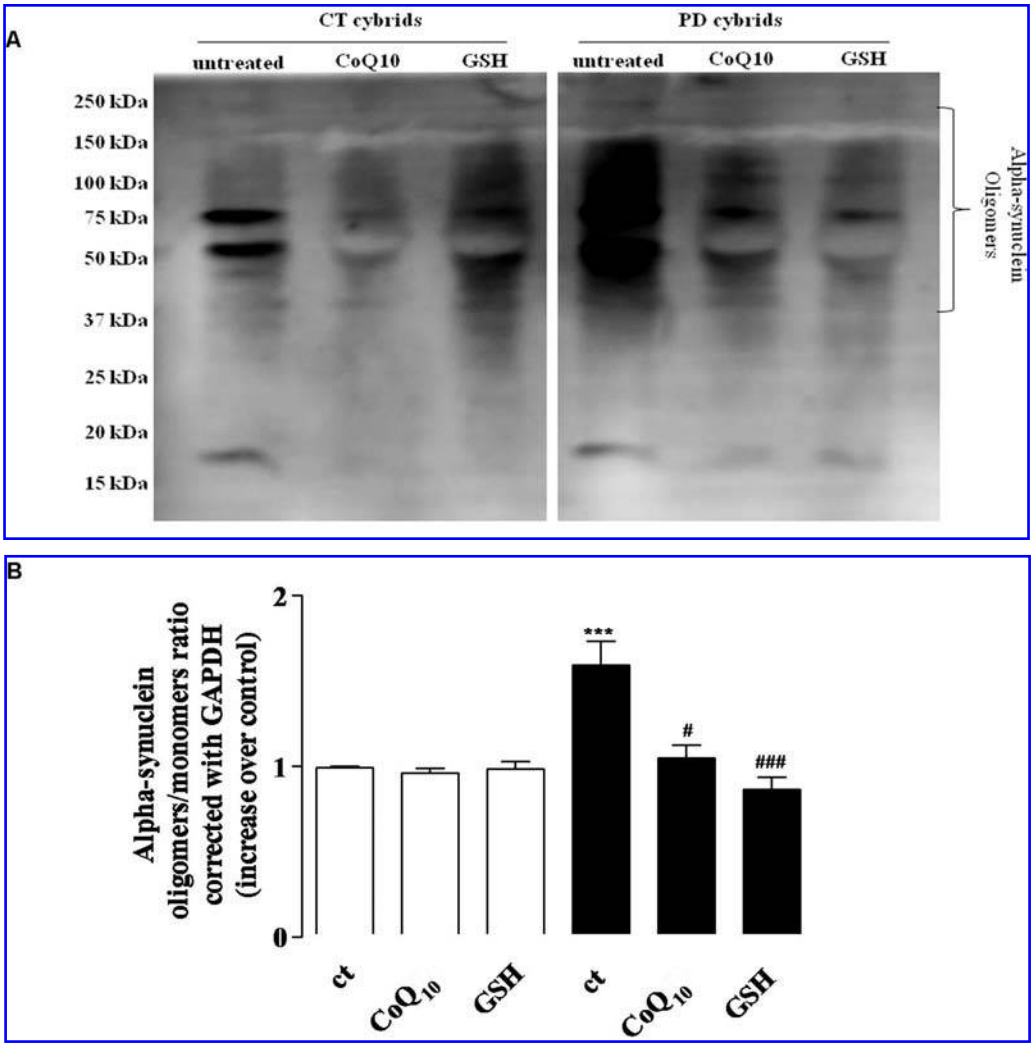

FIG. 3. CoQ_{10} and GSH effect on α-synuclein oligomerization. (**A**) PAGE analysis demonstrated that oligomeric α-synuclein in PD cybrids was reduced in the presence of antioxidants. Western blot is representative of six independent experiments. (**B**) The ratio between the densitometry of α-synuclein oligmers and monomer levels corrected with GAPDH. ***$p <$ 0.001, significantly different when compared with untreated CT cybrids. #$p < 0.05$, ###$p < 0.001$ significantly different when compared with untreated PD cybrids. *White bars*, CT cybrids; *black bars*, PD cybrids.

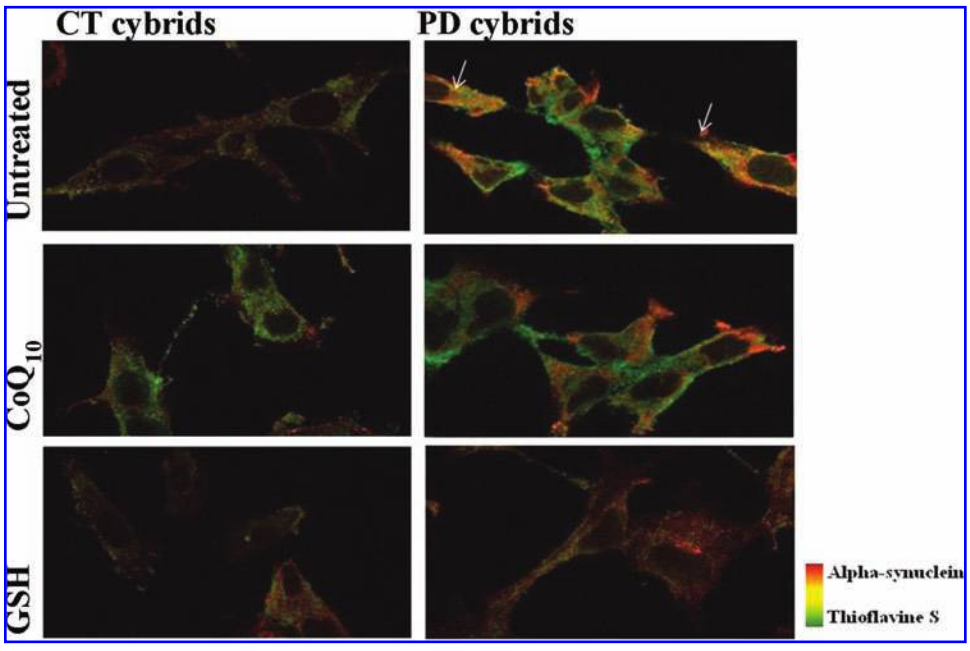

FIG. 4. CoQ_{10} and GSH effect on Lewy bodies "like" formation in PD cybrids. Increased colocalization of α-synuclein with thioflavin S in PD cybrids was reversed by antioxidants. *White arrows,* focal regions of increased α-synuclein β-sheet content. The figure is representative of two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

idants. For that reason, we measured changes in protein carbonyl and sulfhydryl groups. Our results showed an increase in carbonyl content and a decrease in sulfhydryl content in untreated PD cybrids as compared with untreated CT cybrids (Fig. 2). We observed that exposure to CoQ_{10} or GSH decreases carbonyl content in PD cybrids (Fig. 2A). However, only CoQ_{10} was able to prevent the decrease in protein sulfhydryl groups in PD cybrids. GSH was ineffective, indicating that this concentration was not enough to catalyze the reduction of disulfide bridges in proteins (Fig. 2B). We also detected that immunoprecipitated α-synuclein was oxidated in PD cybrids, showing an increase in carbonyl groups (Fig. 2C).

CoQ_{10} and GSH treatment decreases α-synuclein oligomerization in PD cybrids

One of the biochemical pathways that may lead to protein aggregation is oxidative stress. *In vitro* oxidation and nitration of α-synuclein results in cross-linking of α-synuclein, forming stable high-molecular-mass α-synuclein aggregates (37). We previously showed that untreated PD cybrids have enhanced α-synuclein oligomers when compared with untreated CT cybrids (14). Therefore, to address the role of oxidative stress in α-synuclein oligomerization, we demonstrate that CoQ_{10} and partially GSH decreased α-synuclein oligomer content in PD cybrids (Fig. 3A). Moreover, antioxidants did not induce differences in CT cybrids. We evaluated the ratio between α-synuclein oligomers and monomers to prove that this difference is not a result of an increased presence of α-synuclein in PD cybrids (Fig. 3B). As previously described by our group, thioflavin S staining colocalized more with α-synuclein immunochemical staining in PD cybrids (14). After CoQ_{10} and GSH treatment, we observed a decrease in this colocalization (Fig. 4).

The role of ROS in PD cybrids microtubule alterations

ROS can interact with a variety of macromolecules, such as proteins. Consequently, it can interact with tubulin, interfering with microtubules dynamics. We previously showed that mitochondrial dysfunction *via* ATP depletion can lead to microtubule disruption by an increased

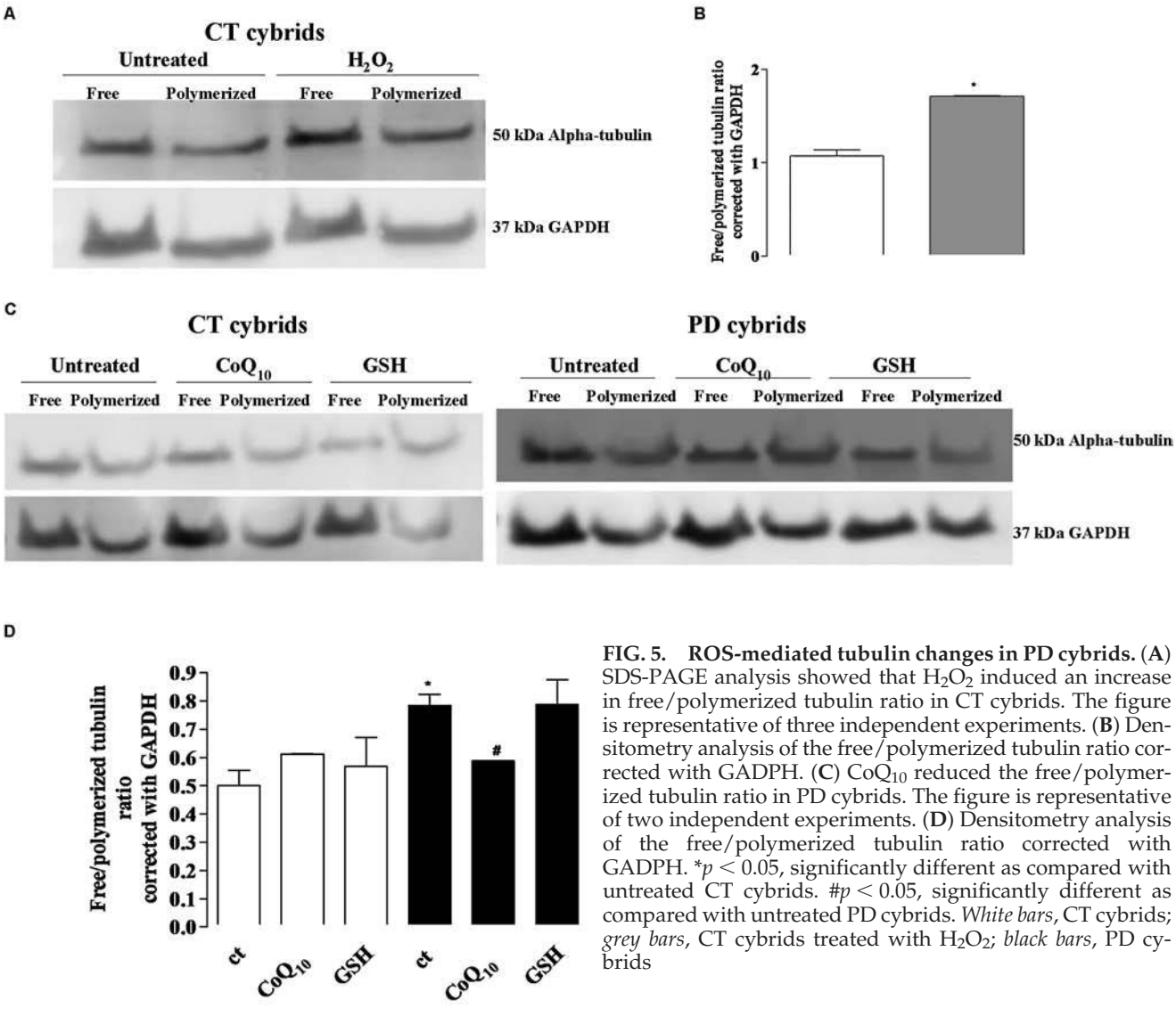

FIG. 5. ROS-mediated tubulin changes in PD cybrids. (**A**) SDS-PAGE analysis showed that H_2O_2 induced an increase in free/polymerized tubulin ratio in CT cybrids. The figure is representative of three independent experiments. (**B**) Densitometry analysis of the free/polymerized tubulin ratio corrected with GADPH. (**C**) CoQ_{10} reduced the free/polymerized tubulin ratio in PD cybrids. The figure is representative of two independent experiments. (**D**) Densitometry analysis of the free/polymerized tubulin ratio corrected with GADPH. *$p < 0.05$, significantly different as compared with untreated CT cybrids. #$p < 0.05$, significantly different as compared with untreated PD cybrids. *White bars*, CT cybrids; *grey bars*, CT cybrids treated with H_2O_2; *black bars*, PD cybrids

free/polymerized tubulin ratio and therefore induce α-synuclein oligomerization (14).

After the GSH treatment, the free/polymerized tubulin ratio increase was not prevented in PD cybrids. However, CoQ_{10} treatment decreased this ratio significantly, indicating that this antioxidant had a protective effect in microtubule dynamics (Fig. 5C, D). To address ROS involvement in alteration of microtubules, we determined the H_2O_2 effect on the free/polymerized tubulin ratio in CT cybrids. As expected, CT cybrids exposed to H_2O_2 showed a higher free/polymerized tubulin ratio as compared with untreated CT cybrids (Fig. 5A, B).

CoQ_{10} but not GSH improves mitochondrial function

CoQ_{10} is a cofactor of the electron-transporter chain, and GSH helps to modulate cell responses to oxidative stress. To determine whether these two antioxidants improve mitochondrial function in PD cybrids, we evaluated complex I activity of mitochondrial respiratory chain and measured ATP levels after CoQ_{10} or GSH treatment. Interestingly, CoQ_{10} improved complex I activity and subsequently sustained ATP levels in PD cybrids, whereas GSH has no effect on either complex I activity or ATP levels (Fig. 6). Antioxidant treatments in CT cybrids did not change complex I activity and ATP levels.

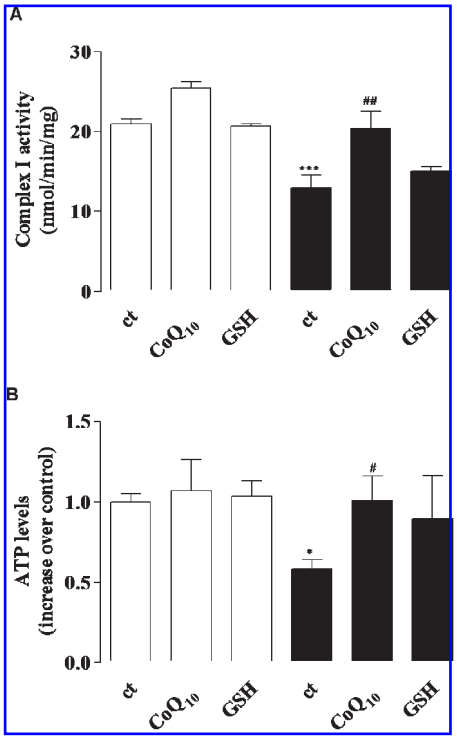

FIG. 6. CoQ_{10} and GSH effect on mitochondrial function. (**A**) Mitochondrial respiratory chain complex I activity in PD cybrids was improved by CoQ_{10} treatment. Complex I activity was determined by using CoQ_1 as substrate and expressed in nmol/min/mg of protein, as the mean ± SEM from five different experiments. (**B**) ATP levels in PD cybrids were ameliorated by CoQ_{10} treatment. Cell ATP levels were determined with HPLC and expressed as a percentage of untreated CT cybrids, with the mean ± SEM from three different experiments. $^*p < 0.05$; $^{***}p < 0.001$, significantly different as compared with untreated CT cybrid. $\#p < 0.05$; $\#\#p < 0.01$, significantly different as compared with untreated PD cybrids. *White bars*, CT cybrids; *black bars*, PD cybrids.

Discussion

In this report, we described for the first time that oxidative stress induced by inherited mitochondrial dysfunction contributes to α-synuclein oligomerization. Furthermore, we proved that antioxidants efficiently decreased α-synuclein oligomer content because of mitochondrial function improvement or by preventing protein carbonylate formation (Fig. 7). Mitochondrial dysfunction is thought to play an important role in the pathogenesis of idiopathic PD (22, 34). PD has been also associated with an increase in oxidative stress, and the fact that age is a key risk factor provides considerable support for the free radical hypothesis (2, 20). Hence, changes in mitochondrial function, oxidative stress, as well as changes in α-synuclein conformation leading to its aggregation and posttranslational modifications, are thought to be key pathogenetic events in PD (3, 6). As a result, elucidation of the cellular mechanisms that influence changes occurring in α-synuclein biochemistry leading to its aggregation and consequent neuronal dysfunction may help in understanding PD pathogenesis.

We observed that mtDNA-driven complex I defect leads to an increase in ROS formation (Fig. 1). ROS can also arise from mutation-altered or damaged metalloenzymes involved in oxidative metabolism (33). Oxidative stress is defined as the result of an imbalance between excessive production of ROS and limited antioxidant defenses. When this imbalance occurs and progresses, damage of a wide variety of important macromolecules occurs, leading to cell death (29). We demonstrated that by increasing antioxidants within the cells, ROS production decreased (Fig. 1). Mitochondria generate most of the ROS as a by-product of oxidative phosphorylation. Moreover, mitochondrial dysfunction and consequent impairment of ATP generation may contribute to the overproduction of ROS (13). At present, the significant role of antioxidants in the prevention or treatment of PD is still controversial. We report that antioxidants, by preventing protein oxidation and/or improving mitochondrial function, rescue α-synuclein oligomerization (Fig. 3). Because PD is a protein conformational disease, the pathogenic dysfunctional aggregation of proteins in nonnative conformations (α-synuclein) may be associated with metabolic derangements and excessive production of ROS. Free radicals derived from mitochondrial dysfunction can interact with protein side chains (19). In Fig. 2, we show that ROS induce protein carbonylation and the loss of protein thiols. More interesting is that we demonstrated that α-synuclein is carbonylated in PD cybrids (Fig. 2C). Several observations support the hypothesis that modifications associated with oxidative stress–mediated posttranslational modifications are involved in altering the biophysical properties of α-synuclein and promoting the aggregation of this protein (21, 37, 44). This α-synuclein oxidation seems to potentiate its oligomerization (Fig. 3) and increased β-sheet content (Fig. 4).

The steps preceding α-synuclein fibril formation include a conformational change from α-helix to β-pleated sheet. Thus, oxidative stress modifications may result in covalent

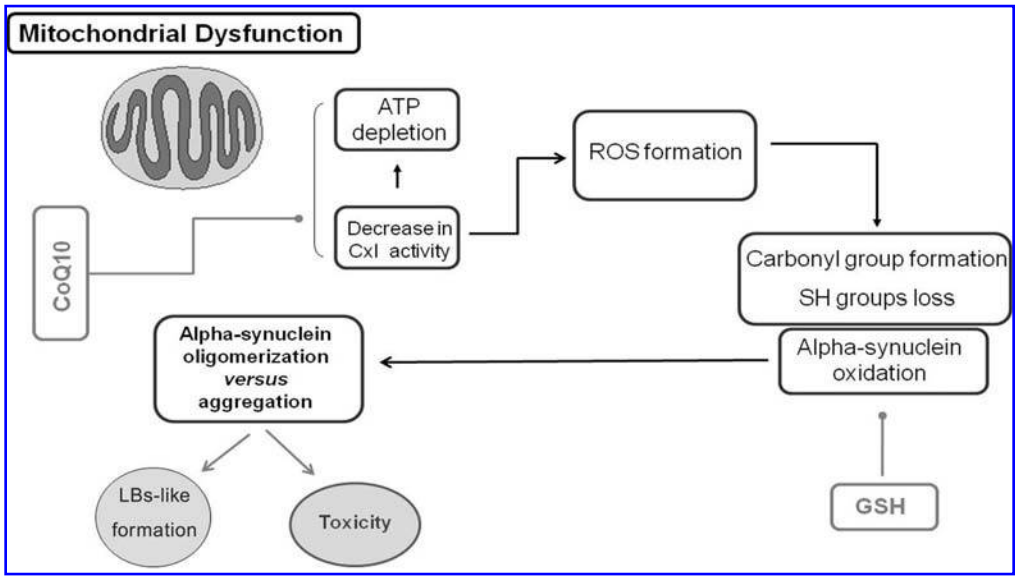

FIG. 7. Schematic illustration describes a sequence of pathologic events induced by mitochondrial dysfunction. PD cybrids with sporadic PD patient's mitochondrial DNA show a decrease in the activity of mitochondrial complex I. This decrease leads to an increase in ROS production and to a reduction in ATP levels. ROS formation potentiates protein oxidation (α-synuclein oxidation). This posttranslational modification of α-synuclein seems to be, at least in part, responsible for its oligomerization and aggregation. Moreover, ROS seem to contribute to microtubule disruption, although this effect is more correlated with a decrease in ATP levels. Our model implies that mitochondria-dependent ROS production potentiates α-synuclein oligomerization in sporadic PD.

cross-linkings and stabilization of α-synuclein filaments, leading to α-synuclein protofibrils and fibrils formation (3). Interestingly, we observed that both CoQ_{10} and GSH decreased α-synuclein oligomer content (Figs. 3 and 4). Accordingly, antioxidant compounds show potent anti–α-synuclein fibrillogenic and α-synuclein fibril-destabilizing effects, which is in accordance with the enhancement of α-synuclein fibril formation by oxidation (30). Thus, generation of ROS is likely to exacerbate the aggregation process that initially forms oligomeric species that self-assemble into fibrillar structures. This process of fibrillization is important to the formation of LB, as these organized structures became deposited (9). Thus, our results provide evidence that mitochondria-mediated oxidative stress induces α-synuclein oligomerization through protein oxidation. The two antioxidants used in this work act in different cellular targets. CoQ_{10} acts as an antioxidant and also improves mitochondrial function (45) by increasing complex I activity and ATP levels (Fig. 6). GSH acts as an antioxidant only by decreasing ROS formation and protein carbonyl content (Figs. 1 and 2). We previously described that inherent mitochondrial dysfunction in PD cybrids leads to α-synuclein oligomerization *via* ATP depletion and subsequent microtubule disruption, which in turn induces tubulin–α-synuclein interaction (14).

By treating cells with CoQ_{10}, that rescues ATP levels, the ratio free/polymerized tubulin decreased in PD cybrids (Fig. 5). Conversely, GSH treatment did not rescue mitochondria-mediated microtubule disruption. As a control, we used H_2O_2 to investigate CT cybrids response on oxidative stress and observed that CT cybrids after H_2O_2 treatment revealed an increased in free tubulin, as compared with untreated CT cybrids (Fig. 5A and B).

A dual relation exists between α-synuclein and the production of free radicals. Not only can the oxidative process transform nonaggregated α-synuclein into its aggregated form, but also may be able to generate H_2O_2 *in vitro* by itself (43). Therefore, it could be speculated that once an oxidation process starts, an enhancement of α-synuclein aggregation occurs, leading to an increased neurotoxicity, with release of cellular iron, which catalyzes further oxidations, initiating a positive feedback of oxidative neurodegeneration. Moreover, high expression levels of the *wt* and mutant forms of α-synuclein cause an increase in ROS production (28). Also important is the fact that α-synuclein toxicity in cultured neurons is blocked by antioxidants (23).

The data presented here show that, by increasing cellular antioxidants, proteins do not became oxidized, which prevents α-synuclein oligomerization and, what is more striking, also avoid the positive feedback of free radical (H_2O_2) generation, which in turn can alter microtubule networks. Therefore, we propose that mitochondrial ROS potentiate α-synuclein aggregation because of protein oxidation, and that antioxidant administration early in life may have benefits.

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Abbreviations

CoQ_{10}, coenzyme Q10; CT cybrids, control cybrids; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; LB, Lewy body; PD, Parkinson disease; ROS, reactive oxygen species.

Disclosure Statement

No competing financial interests exist.

References

1. Alim MA, Hossain MS, Arima K, Takeda K, Izumyama Y, Nakamura M, Kaji H, Shinoda T, Hisanaga S, and Uéda K. Tubulin seeds alpha-synuclein fibril formation. *J Biol Chem* 277: 2112–2117, 2002.
2. Benzi G and Moretti A. Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic Biol Med* 19: 77–101, 1995.
3. Beyer K. Alpha-synuclein structure, posttranslational modification and alternative splicing as aggregation enhancers. *Acta Neuropathol* 112: 237–251, 2006.
4. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
5. Cappeleti G, Surrey T, and Maci R. The parkinsonism producing neurotoxin MPP^+ affects microtubule dynamics by acting as a destabilizing factor. *FEBS Lett* 579: 4781–4786, 2005.
6. Cardoso SM, Moreira PI, Agostinho P, Pereira C, and Oliveira CR. Neurodegenerative pathway in Parkinson's disease: therapeutical strategies. *Curr Drugs Targets-CNS&ND* 4: 405–419, 2005.
7. Cardoso SM, Santana I, Swerdlow RH, and Oliveira CR Mitochondrial dysfunction of Alzheimer's disease cybrids enhances Abeta toxicity. *J Neurochem* 89: 1417–1426, 2004.
8. Cassarino DS, Fall CP, Swerdlow RH, Smith TS, Halvorsen EM, Miller SW, Parks JP, Parker WD Jr, and Bennett JP Jr. Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. *Biochim Biophys Acta* 1362: 77–86, 1997.
9. Cookson MR and Brug M. Cell systems and the toxic mechanism(s) of alpha-synuclein. *Exp Neurol* 1: 5–11, 2007.
10. Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, and Marsden CD. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* 52: 381, 1989.
11. Di Monte D, Ross D, Bellomo G, Eklow L, and Orrenius S. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* 235: 334–342, 1984.
12. Dickson DW, Liu W, Hardy J, Farrer M, Mehta N, Uitti R, Mark M, Zimmerman T, Golbe L, Sage J, Sima A, D'Amato C, Albin R, Gilman S, and Yen SH. Widespread alterations of alpha-synuclein in multiple system atrophy. *Am J Pathol* 155: 1241–1251, 1999.
13. Domingues AF, Arduíno DM, Esteves ARF, Swerdlow RH, Oliveira CR, and Cardoso SM. Mitochondria and ubiquitin-proteasomal system interplay: relevance to Parkinson's disease. *Free Radic Biol Med* 45: 820–825, 2008.
14. Esteves ARF, Domingues AF, Arduíno D, Nunes-Correia I, Januário C, Swerdlow RH, Oliveira CR, and Cardoso SM. Mitochondrial dysfunction towards protein aggregation in Parkinson's disease: contribution of microtubule depolymerization. *Soc Neurosci Abstracts* S7: 370.7, 2007.
15. Esteves ARF, Domingues AF, Ferreira IL., Januário C, Swerdlow RH, Oliveira CR, and Cardoso SM. Mitochondrial dysfunction occurs in Parkinson's disease cybrids containing an nt2 neuron-like nuclear background. *Mitochondrion* 8: 219–228, 2008.
16. Floor E and Wetzel MG. Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. *J Neurochem* 70: 268, 1998.
17. Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55: 259, 1996.
18. Gelb DJ, Oliver E, and Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 56: 33–39, 1999.
19. Giasson BI, Ischiropoulos H, Lee VM-Y, and Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radic Biol Med* 32: 1264–1275, 2002.
20. Halda A and Lotharius J. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? *Exp Neurol* 193: 279–290, 2005.
21. Hashimoto M, Hsu LJ, Xia Y, Takeda A, Sisk A, Sundsmo M, and Masliah E. Oxidative stress induces amyloid-like aggregate formation of NACP/α-synuclein in vitro. *Neuroreport* 10: 717–721, 1999.
22. Hattori N, Tanaka M, Ozawa T, and Mizuno Y. Immunohistochemical studies on complexes I, II, III, IV of mitochondria in Parkinson's disease. *Ann Neurol* 30: 563–571, 1991.
23. Kang JH and Kim KS. Enhanced oligomerization of the alpha-synuclein mutant by the Cu,Zn-superoxide dismutase and hydrogen peroxide system. *Mol Cells* 15: 87–93, 2003.
24. Kim M, Jung W, Lee IH, Bhak G, Paik SR, and Hahn JS. Impairment of microtubule system increases alpha-synuclein aggregation and toxicity. *Biochem Biophys Res Commun* doi:10.1016/j.bbrc.2007.11.020, 2007.
25. Krige D, Carrol MT, Cooper JM, Marsden CD, and Schapira AHV. Platelet mitochondrial function in Parkinson's disease: the Royal Kings and Queens Parkinson Disease Research Group. *Ann Neurol* 32: 782–788, 1992.
26. Levine RL, Williams JA, Stadtman ER, and Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 233: 346–357, 1994.
27. Mårtensson J, Lai JC, and Meister A. High-affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function. *Proc Natl Acad Sci U S A* 87: 7185–7189, 1990.
28. Moussa CE, Wersinger C, Tomita Y, and Sidhu A. Differential cytotoxicity of human wild type and mutant alpha-synuclein in human neuroblastoma SH-SY5Y cells in the presence of dopamine. *Biochemistry* 43: 5539–5550, 2004.
29. Nunomura A, Moreira PI, Lee HG, Zhu X, Castellani RJ, Smith MA, and Perry G. Neuronal death and survival under oxidative stress in Alzheimer and Parkinson diseases. *CNS Neurol Disord Drug Targets* 6: 411–423, 2007.
30. Ono K and Yamada M. Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. *J Neurochem* 97: 105–115, 2006.
31. Ragan CI, Wilson MT, Darley-Usmar VM, and Lowe PN. Subfractionation of mitochondria, and isolation of the proteins of oxidative phosphorylation. In *Mitochondria, A Practical Approach* edited by Darley-Usmar VH, Rickwood D, and Wilson HT. London: IRL Press, 1987, pp. 79–112.

32. Ren Y and Feng J. Rotenone selectively kills serotonergic neurons through a microtubule-dependent mechanism. *J Neurochem* 103: 303–311, 2007.
33. Sayre LM, Moreira PI, Smith MA, and Perry G. Metal ions and oxidative protein modification in neurological disease. *Ann Ist Super Sanità* 41: 143–164, 2005.
34. Schapira A, Cooper JM, Dexter D, Clark JB, Jenner P, and Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 54: 823–827, 1990.
35. Schulz JB, Lindenau J, Seyfried J, and Dichgans J. Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem* 267: 4904, 2000.
36. Shults CW, Haas RH, Passov D, and Beal MF. Coenzyme Q(10) levels correlate with the activities of complexes I and II/III in mitochondria from parkinsonian and non-parkinsonian subjects. *Ann Neurol* 42: 261–264, 1997.
37. Souza JM, Giasson BI, Chen Q, Lee VM, and Ischiropoulos H. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers: implications of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* 275: 18344–18349, 2000.
38. Stocchi V, Cucchiarini L, Chiarantini L, Palma P, and Crescentini G. Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal Biochem* 146:118–124, 1985.
39. Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Maguire RS, Bennett JP Jr, Davis RE, and Parker WD Jr. Cybrids in Alzheimer's disease: a cellular model of the disease? *Neurology* 49: 918–925, 1997.
40. Swerdlow RH, Parks JK, Miller SW, Tuttle JB, Trimmer PA, Sheehan JP, Bennett JP Jr, Davis RE, and Parker WD Jr. Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann Neurol* 40: 663–671, 1996.
41. Takahashi M, Ko LW, Kulathingal J, Jiang P, Sevlever D, and Yen SH. Oxidative stress-induced phosphorylation, degradation and aggregation of alpha-synuclein are linked to upregulated CK2 and cathepsin D. *Eur J Neurosci* 26: 863–874, 2007.
42. Trimmer PA, Borland MK, Keeney PM, Bennett JP Jr, and Parker WD Jr. Parkinson's disease transgenic mitochondrial cybrids generate Lewy inclusion bodies. *J Neurochem* 88: 800–812, 2004.
43. Turnbull S, Tabner BJ, El-Agnaf OM, Moore S, Davies, Y, and Allsop D. Alpha-synuclein implicated in Parkinson's disease catalyses the formation of hydrogen peroxide in vitro. *Free Radic Biol Med* 30: 1163–1170, 2001.
44. Uversky VN. Neuropathology, biochemistry, and biophysics of α-synuclein aggregation. *J Neurochem* doi:10.1111/j.1471–4159.2007.04764, 2007.
45. Winkler-Stuck K, Wiedemann FR, Wallesch CW, and Kunz WS. Effect of coenzyme Q10 on the mitochondrial function of skin fibroblasts from Parkinson patients. *J Neurol Sci* 220: 41–48, 2004.
46. Ziegler DM. Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem* 54: 305–329, 1985.

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2. Rita Perfeito, Teresa Cunha-Oliveira, Ana Cristina Rego. 2012. Revisiting oxidative stress and mitochondrial dysfunction in the pathogenesis of Parkinson disease—resemblance to the effect of amphetamine drugs of abuse. *Free Radical Biology and Medicine* **53**:9, 1791-1806. [CrossRef]
3. María Morán, David Moreno-Lastres, Lorena Marín-Buera, Joaquín Arenas, Miguel A. Martín, Cristina Ugalde. 2012. Mitochondrial respiratory chain dysfunction: Implications in neurodegeneration. *Free Radical Biology and Medicine* **53**:3, 595-609. [CrossRef]
4. D. M. Arduino, A. Raquel Esteves, L. Cortes, D. F. Silva, B. Patel, M. Grazina, R. H. Swerdlow, C. R. Oliveira, S. M. Cardoso. 2012. Mitochondrial metabolism in Parkinson's disease impairs quality control autophagy by hampering microtubule-dependent traffic. *Human Molecular Genetics* . [CrossRef]
5. Marina S Gorbatyuk, Arseniy Shabashvili, Weijun Chen, Craig Meyers, Layla F Sullivan, Max Salganik, Jonathan H Lin, Alfred S Lewin, Nicholas Muzyczka, Oleg S Gorbatyuk. 2012. Glucose Regulated Protein 78 Diminishes #-Synuclein Neurotoxicity in a Rat Model of Parkinson Disease. *Molecular Therapy* **20**:7, 1327-1337. [CrossRef]
6. D. Santos, S.M. Cardoso. 2012. Mitochondrial dynamics and neuronal fate in Parkinson's disease. *Mitochondrion* **12**:4, 428-437. [CrossRef]
7. Russell H. Swerdlow . 2012. Does Mitochondrial DNA Play a Role in Parkinson's Disease? A Review of Cybrid and Other Supportive Evidence. *Antioxidants & Redox Signaling* **16**:9, 950-964. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
8. S.M. Cardoso, A.R. Esteves, D.M. Arduíno. 2012. Mitochondrial Metabolic Control of Microtubule Dynamics Impairs the Autophagic Pathway in Parkinsons Disease. *Neurodegenerative Diseases* **10**:1-4, 38-40. [CrossRef]
9. Sonia Gandhi, Andrey Y. Abramov. 2012. Mechanism of Oxidative Stress in Neurodegeneration. *Oxidative Medicine and Cellular Longevity* **2012**, 1-11. [CrossRef]
10. Yuxing Zhang , Yanzhi Du , Weidong Le , Kankan Wang , Nelly Kieffer , Ji Zhang . 2011. Redox Control of the Survival of Healthy and Diseased Cells. *Antioxidants & Redox Signaling* **15**:11, 2867-2908. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
11. Ester López-Gallardo, Ruth Iceta, Eldris Iglesias, Julio Montoya, Eduardo Ruiz-Pesini. 2011. OXPHOS toxicogenomics and Parkinson's disease. *Mutation Research/Reviews in Mutation Research* . [CrossRef]
12. Christelle En Lin Chua, Bor Luen Tang. 2011. Rabs, SNAREs and #-synuclein — Membrane trafficking defects in synucleinopathies. *Brain Research Reviews* **67**:1-2, 268-281. [CrossRef]
13. A. R. Esteves, D. M. Arduíno, D. F. F. Silva, C. R. Oliveira, S. M. Cardoso. 2011. Mitochondrial Dysfunction: The Road to Alpha-Synuclein Oligomerization in PD. *Parkinson's Disease* **2011**, 1-20. [CrossRef]
14. Micronutrients for the Prevention and Improvement of the Standard Therapy for Parkinson’s Disease 197-220. [CrossRef]
15. S.M. Cardoso, C.F. Pereira, P.I. Moreira, D.M. Arduino, A.R. Esteves, C.R. Oliveira. 2010. Mitochondrial control of autophagic lysosomal pathway in Alzheimer's disease. *Experimental Neurology* **223**:2, 294-298. [CrossRef]
16. Jin-zhong Huang, Ying-zhu Chen, Min Su, Hui-fen Zheng, Ya-ping Yang, Jing Chen, Chun-Feng Liu. 2010. dl-3-n-Butylphthalide prevents oxidative damage and reduces mitochondrial dysfunction in an MPP+-induced cellular model of Parkinson's disease. *Neuroscience Letters* **475**:2, 89-94. [CrossRef]
17. A. Raquel Esteves, Jane Lu, Mariana Rodova, Isaac Onyango, E Lezi, Richard Dubinsky, Kelly E. Lyons, Rajesh Pahwa, Jeffrey M. Burns, Sandra M. Cardoso, Russell H. Swerdlow. 2010. Mitochondrial respiration and respiration-associated proteins in cell lines created through Parkinson’s subject mitochondrial transfer. *Journal of Neurochemistry* **113**:3, 674-682. [CrossRef]
18. A. Raquel Esteves, Daniela M. Arduíno, Russell H. Swerdlow, Catarina R. Oliveira, Sandra M. Cardoso. 2010. Dysfunctional mitochondria uphold calpain activation: Contribution to Parkinson's disease pathology. *Neurobiology of Disease* **37**:3, 723-730. [CrossRef]
19. Paula I. Moreira, Xiongwei Zhu, Xinglong Wang, Hyoung-gon Lee, Akihiko Nunomura, Robert B. Petersen, George Perry, Mark A. Smith. 2010. Mitochondria: A therapeutic target in neurodegeneration. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1802**:1, 212-220. [CrossRef]

20. Patricia A. Trimmer, James P. Bennett. 2009. The cybrid model of sporadic Parkinson's disease. *Experimental Neurology* **218**:2, 320-325. [CrossRef]
21. Chun-Yi Liu, Cheng-Feng Lee, Yau-Huei Wei. 2009. Role of Reactive Oxygen Species-elicited Apoptosis in the Pathophysiology of Mitochondrial and Neurodegenerative Diseases Associated With Mitochondrial DNA Mutations. *Journal of the Formosan Medical Association* **108**:8, 599-611. [CrossRef]
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