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Mutant and Wild-Type α-Synuclein Interact with Mitochondrial Cytochrome C Oxidase

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Abstract

α-synuclein, a presynaptic protein, was found to be the major component in the Lewy bodies (LB) in both inherited and sporadic Parkinson's disease (PD). Furthermore, rare mutations of α-synuclein cause autosomal-dominant PD. However, it is unknown how α-synuclein is involved in the pathogenesis of nigral degeneration in PD. In this study, we examine the protein-protein interactions of wild-type and mutant (A53T) α-synuclein with adult human brain cDNA expression library using the yeast two-hybrid technique. We found that both normal and mutant α-synuclein specifically interact with the mitochondrial complex IV enzyme, cytochrome C oxidase (COX). Wild-type and mutant α-synuclein genes were further fused with c-Myc tag and translated in rabbit reticulocyte lysate. Using anti-c-Myc antibody, we demonstrated that both wild-type and mutant α-synuclein, coimmunoprecipitated with COX. We also showed that potassium cyanide, a selective COX inhibitor, synergistically enhanced the sensitivity of SH-SY5Y neuroblastoma cells to dopamine-induced cell death. In conclusion, we found specific protein-protein interactions of α-synuclein, a major LB protein, to COX, a key enzyme of the mithochondrial respiratory system. This interaction suggests that α-synuclein aggregation may contribute to enhance the mitochondrial dysfunction, which might be a key factor in the pathogenesis of PD.

Index Entries: α-synuclein; coimmunoprecipitation; cytochrome c oxidase; in vitro translation; Parkinson's disease; potassium cyanide; two-hybrid system.

Introduction

One of the neuropathological hallmarks of Parkinson's disease (PD) is the appearance of intracytoplasmatic inclusions termed Lewy bodies (LB), found particularly, but not exclusively, in the substantia nigra (SN) (Forno 1996). α-synuclein, a 140 amino acid presynaptic protein of yet unknown function, was found to be the major component in the LB in both inherited and sporadic PD (Spillantini et al., 1997, 1998; Baba et al., 1998). Two missense mutations in the α-synuclein gene, i.e., G209A (Ala53Thr) and G88C (Ala30Pro), were shown to cause a rare autosomal dominant form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). Intracellular accumulation of α-synuclein was also found in other neurodegenerative diseases, such as Alzheimer's disease (Ueda et al., 1993), dementia with LB (Lantos et al., 1994), and multisystem atrophy (Mezey et al., 1998; Tu et al., 1998). The role played by wild-type and mutant forms of α-synuclein in neurodegeneration in general and in nigral cell death in PD in particular, is unclear. In vitro studies revealed that α-synuclein tend to self-aggregate and that this phenomenon increases in the mutant forms (Narhi et al., 1999; Conway et al., 1998; Bennet et al., 1999). Further studies indicated that α-synuclein inhibits the activities of phospholipase D2, protein kinase C

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(PKC), and the production of phosphatidic acid (Jenco et al., 1998; Ostrerova et al.1999). Mice lacking α-synuclein exhibit normal brain architecture and normal dopaminergic neurons but display reduction in striatal DA and accelerated recovery of DA release when exposed to multiple stimuli (Abeliovich et al., 2000). In contrast, transgenic mice expressing human wild-type and mutant α-synuclein display features of α-synucleinopathy including LB-like pathology, neuronal degeneration, axonal damage, and motor defects (Masliah et al., 2000; van der Putten et al., 2000; Kahle et al., 2000). A recent study showed that α-synuclein interacts with another protein, synphilin-1 (Engelender et al., 1999). However, it is not unlikely that α-synuclein might interact and interfere with function of other cellular proteins. We therefore studied the protein-protein interactions of wild-type or mutant (A53T) α-synuclein with proteins expressed in adult human brain by the yeast two-hybrid system using adult human brain expression library.

Methods

DNA Constructs and Mutagenesis

Wild-type α-synuclein cDNA clone was prepared from total RNA extracted from a post mortem sample of normal human brain. Reverse transcriptase polymerase chain reaction (RT-PCR) reactions were carried out using a RT-PCR kit (Promega, Madison, WI) with the following primers: P7 (5′ end primer): CGTC TAGAAATTCATTAGCCATGGAT, introducing an XbaI site. RP413 (3′ end primer): CTGCGGCCGC GAGCAAAGATATTTCTTA, introducing a NotI site. The reaction product was purified and digested with the restriction enzymes XbaI and NotI. The restricted DNA was ligated into the pcDNA3.1(-) vector (Invitrogene, San Diego CA), which was then introduced into the DH5α bacterial cells and grown on amphicilin containing plates. Positive clones were isolated and sequenced to confirm their identity.

Mutant (A53T) α-synuclein was obtained by PCR mutagenesis, using the overlap extension procedure described by Ho et al. (1989). A set of two overlapping primers containing the desired mutation were constructed: P241763 (5′ end primer): CATGGTGT GACAACTGTGGCTGAG, RP241764 (3′ end primer): CTCAGCCACAGTTGTCACACCATG. The outside primers were P7 and RP413, as described earlier. The resulting mutagenized PCR fragment was sequenced to verify the mutation and to ensure that no other mutation occurred during the amplification process. Wild type and A53T mutant α-synuclein genes were further cloned into pAS2-1 yeast expression vector (Clontech, #K1604-B, Palo Alto, CA): pcDNA3.1(-) contain wild-type and A53T mutant α-synuclein were first digested with EcoRV and NheI. The restricted DNA was purified, further digested with NcoI andligated into the pAS2-1 vector that was pre-digested with NcoI and SmaI. Positive clones were isolated and sequenced to confirm their identity.

Yeast Two-Hybrid Screen

Wild-type and A53T mutant α-synuclein genes were cloned, as describe ealier, into the pAS2-1 yeast expression vector (Clontech, #K1604-B) so that they were fused in frame to the GAL4-binding domain. This construct was co-transformed to CG1945 yeast strain (Clontech) with a human brain cDNA expression library (Clontech, #HY4004AH) in which the peptides are fused to the GAL4-activating domain. Positive transformed clones were selected on Trp^- Leu^- His^- medium, supplemented with 12 m*M* 3-amino-triazole. To confirm the interaction, purified library vectors from positively interacting clones were re-co-transformed with pAS2-1 α-synuclein vectors (according to the Clontech manual guide). CD40 and tumor necrosis factor receptor-associated factor (TRAF) were used as a positive control (Cheng et al., 1995; Sato et al., 1995), while Lamin c and TRAF were used as negative controls.

In Vitro Translation and Co-Immunoprecipitation

Rabbit reticulocyte lysate (Promega, #L4151) was used to translate wild-type and A53T mutant α-synuclein and cytochrome c oxidase subunit III (COX_{III}). Using a specific primer set (Clontech, #K1613), we incorporated T7 promoter sequence and c-Myc (Myc) tag to the α-synuclein genes. The relevant segments were amplified by PCR and the products were purified, transcribed, and translated using the Promega Kit in the presence of ^{35}S-Met (22 μCi/50 μL reaction mixture, NEG-772, NEN™ Life Science Products, Inc. Boston, MA). The fused proteins containing the Myc epitope tag were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For co-immunoprecipitation, 5 μL of translation mixtures of wild-type or A53T mutant α-synuclein were incubated with 5 μL of COX_{III} translation mixture for 1 h at 30°C in coimmunoprecipitation buffer (470 μL of 20 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1 m*M* dithiothreitol (DTT), 5 μg/mL aprotinin, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), and 0.1% Tween 20 (v/v). Protein-G agarose beads (10 μL) and c-Myc monoclonal anti-

body (MAb; 10 μL, Clontech) were then added and incubated at 4°C for 2 h with continuous rocking. Samples were washed 3 times with 0.5 mL TBS-Tween 0.1%, the supernatant discarded, and the pellets resuspended in 15 μL SDS-loading buffer. The samples (10 μL) were heated to 80°C for 5 min, centrifuged briefly, and loaded onto a SDS-PAGE 15% mini-gel. Gels were dried at 80°C in a constant vacuum for 1 h and exposed overnight to a phosphorimaging screen (Cyclone). The results were analyzed with Cyclone phosphorimager (Packard, Chicago, IL).

Cell Culture and Viability Assay

SH-SY5Y neuroblstoma cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biological industries, Bet-Haemek, Israel) supplemented with 10% fetal calf serum (FCS) and 1% penicilin/streptomycin/nystatin (Biological industries), maintained at 37°C in a humidified 5% CO_2 incubator. One day prior to the experiment 4×10^4 cells in 100 μL culture medium were plated in a 96-well culture dish (Nunce, Rosklide, Denmark). Cells were incubated for 24 h with increased concentrations of dopamine (RBI # D-019, Natick, MA) and/or potassium cyanide (KCN) (Aldrich, Colombus, WI). Cells were incubated with Alamar blue (Wildflower International Inc. Santa Fe, NM), a fluorometric/colourimetric growth indicator of the oxidation-reduction cellular state, which incorporates into the cells and causes fluorescence changes in response to a reduced environment. Reduction related to growth causes the indicator to change from oxidized form (nonfluorescent, blue) to reduced form (fluorescent, red). Cells were incubated 3 h with 10 μL of Alamar blue at 37°C and the Cell viability was evaluated by measuring the fluorescence intensity using a FLUOstar (BMG LabThechnologies, Offenburg, Germany) with excitation at 544 nm and emission at 590 nm.

DNA Sequences

All constructs were sequenced at either the Tel-Aviv University or the Weizmann Institute sequencing units.

Results

To identify possible protein-protein interactions of α-synuclein, wild type and mutant (A53T) proteins were used as "baits" in a two-hybrid screening using an adult human brain cDNA expression library We found one positive clone that specifically interacted with the mutant (A53T) α-synuclein. The DNA of the positive clone was released and purified. Sequence analysis and comparison using the GenBank database identified this positive cDNA clone to the cytochrome c oxidase subunit III (COX_{III}), of the complex IV of the mitochondrial respiratory chain. The positive clone consisted of 766 (9223-9988) nucleotides out of the 782 nucleotides (9206-9988) of the complete human COX_{III} mtDNA, missing the first 5′ 17 nucleotides (Fig. 1). To confirm the specific interaction, we re-co-transformed the positive cDNA, pACT2 COX_{III}, with pAS2-1 wild-type or mutant (A53T) α-synuclein vectors in a naive CG1945 yeast strain. Deliberate co-transformation of the two vectors revealed that both wild-type and mutant (A53T) α-synuclein, specifically interacted with the positive clone (COX_{III}) and allowed the yeast to grow on the His^- selective medium. As a positive control, we co-transformed the CD40 and TRAF genes, which are known to interact with each other in a two-hybrid systems, while co-transformation of Lamin C and TRAF geneswere used as a negative control. As seen in Fig. 2, besides the positive control, only the wild-type (or mutant) α-synuclein co-transformed with the isolated positive clone (COX_{III}) grew on this selective medium. Yeast cells expressing each of the genes alone and the nonrelated genes (negative control) did not grow on this selective medium. To further confirm the specificity of the interaction between α-synuclein and COX_{III}, we performed a coimmunoprecipitation assay of the examined proteins following translation in the presence of ^{35}S-Met. Mutant and wild-type α-synuclein proteins were first fused to a c-Myc tag. Immunoprecipitation using MAb against c-Myc, revealed that the tagged α-synuclein (wild-type or mutant) could be immunoprecipitated by this antibody, whereas the COX protein could not be directly immunoprecipitated. However, when this antibody was used to immunoprecipitate α-synuclein from a mixture of the in vitro translated proteins (both COX and α-synuclein) two bands that correspond to the wild-type or mutant (A53T) α-synuclein and the COX_{III} were consistently obtained (Fig. 3). These results indicate that α-synuclein can directly interact with COX.

We further examined whether oxidative stress induced by dopamine, as a neurotoxin, may enhance mitochondrial dysfunction, caused by COX_{III} inhibition. PC12 and human neuroblastoma cells (SH-SY5Y) were treated with increasing concentration of dopamine or potassium cyanide (KCN), a complex IV inhibitor, and cell survival was measured by Alamar blue. We found that 0.06 m*M* of dopamine and 4 m*M* of KCN were sublethal

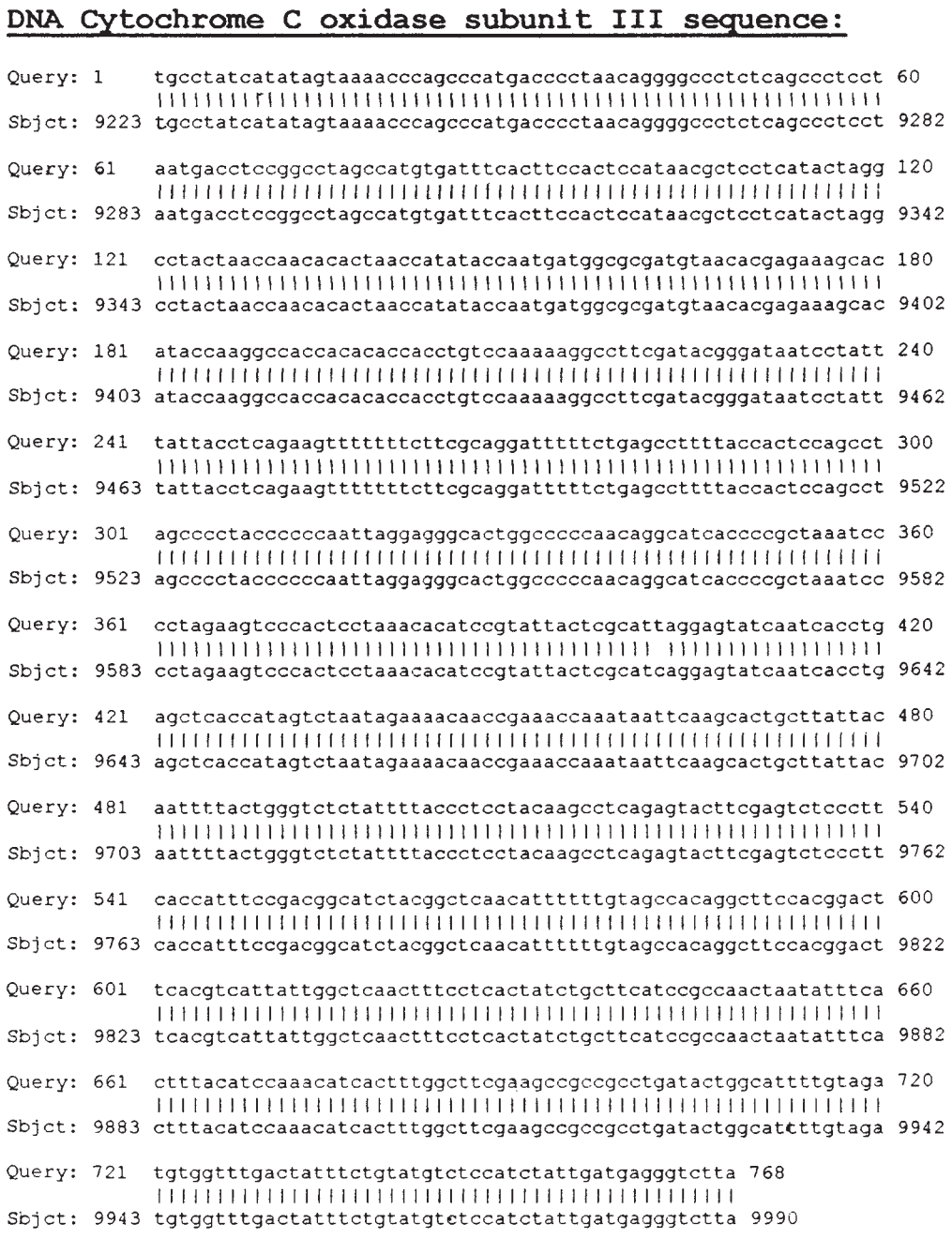

Fig. 1. Identification of the α-synuclein binding protein. DNA sequence of cytochrome C oxidase polypeptide III (COX_{III}); Sbjct refers to the nucleotide sequence of the human mitochondrial COX cDNA, and Query refers to the nucleotide sequence of the positive clone (NCBI gene library).

to the SH-SY5Y cells. However, exposure of the cells for 24 h to sublethal doses of both, dopamine and KCN, showed a toxic effect (26.6%, $p < 0.005$, Fig. 4). Similar results were demonstrated using PC12 cells (data not shown). Thus, combination of dopamine-induced oxidative stress and inhibition of COX_{III} by KCN might cause synergistic toxic effect.

Discussion

The two-hybrid system offered the option to look for the protein-protein interactions of wild-type and

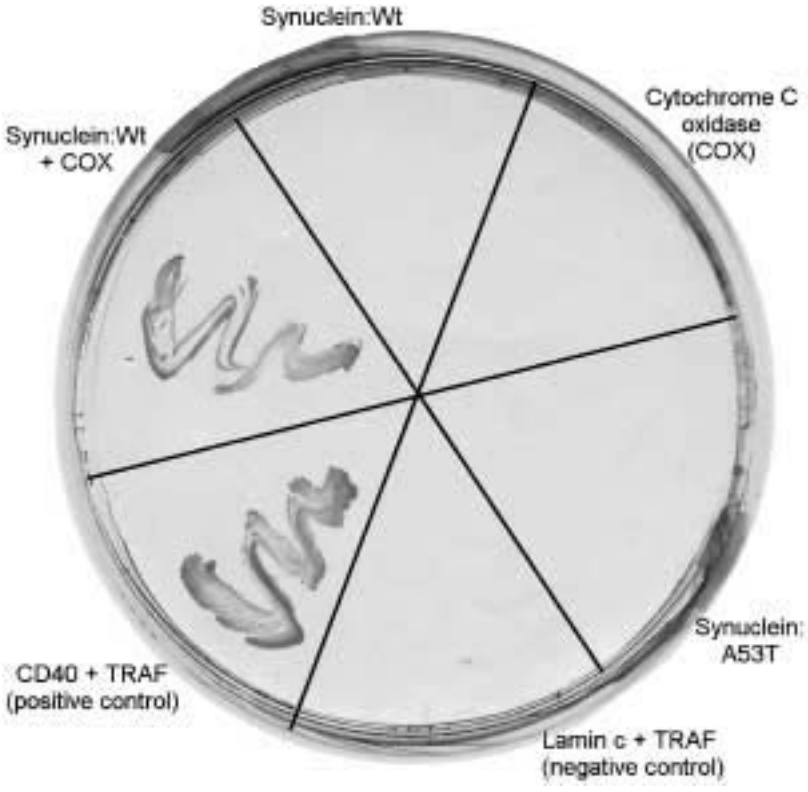

Fig. 2. Protein-protein interaction of α-synuclein with COX_{III} in yeast. Growth of CG-1945 yeast strain (lacking GAL4 with -His, -Trp, -Leu genotype), expressing either Gal4AD-COX, Gal4BD-α-synuclein (wild-type or mutant) or both AD-COX and BD-α-synuclein, on a selective medium (SD: -His, -Trp, -Leu, +12 m*M* tri-amino-triazol), showing specific interaction between α-synuclein and COX_{III}. Negative control, p-laminC-TRAF; positive control, CD40-TRAF.

mutant (A53T) α-synuclein with other cellular proteins. Using this method, we found a specific interaction between α-synuclein and COX_{III} protein, one of 13 proteins that assembles the cytochrome C oxidase complex (complex IV). The specific binding was confirmed by re-co-transformation of the wild-type or mutant α-synuclein genes with the COX_{III} gene to yeast and allowing it to grow on a selective medium. As controls, deliberate co-transformation of yeast two-hybrid system vectors, which code to noninteracting proteins Lamin C and TRAF, did not grow on the selection medium, while the expression of CD40 and TRAF proteins, which are known to interact with each other in other two-hybrid systems, grew. The direct interaction and the relatively high binding between α-synuclein and COX_{III} were also shown by an immunopercipitation assay using antibodies against the c-Myc tag, fused to α-synuclein, which coprecipitated the ^{35}S-labeled COX_{III}.

α-synuclein is a brain specific protein previously identified in synaptophysin-immunoreactive presynaptic terminals and associated with synaptic vesicles (Iwai et al., 1995). The normal function of α-synuclein is unknown but it may play a role in synaptic transmission. The role of α-synuclein in the pathogenesis of PD was demonstrated in rare cases of patients with missense mutations, and the finding that α-synuclein is the major component in LB. Although the exact contribution of α-synuclein to the neurodegeneration is not clear, various studies showed that α-synuclein induced neuronal cell death. It was found that in vitro incubation of full-length α-synuclein and a synthetic peptide fragment of α-synuclein corresponding to residues 61–95 (NAC), both liberate toxic hydroxyl radicals followed by addition of Fe^{+2} (Turnbull et al., 2001). Moreover, overexpression of α-synuclein gene, especially the mutated forms, in neuronal cell lines increased their sensitivity to oxidative stress induced by menadione, hydrogen peroxide, 6-OHDA, MPP^+, and dopamine (Ko et al., 2000; Kanda et al., 2000; Tabrizi et al., 2000; Zhou et al., 2000). In dopaminergic neurons, overexpressing mutant α-synuclein, and oxidative stress induced typical apoptotic cell death (Zhou et al., 2000).

Wild-type α-synuclein was found in intraneuronal LB inclusions, with a large variety of intermediate

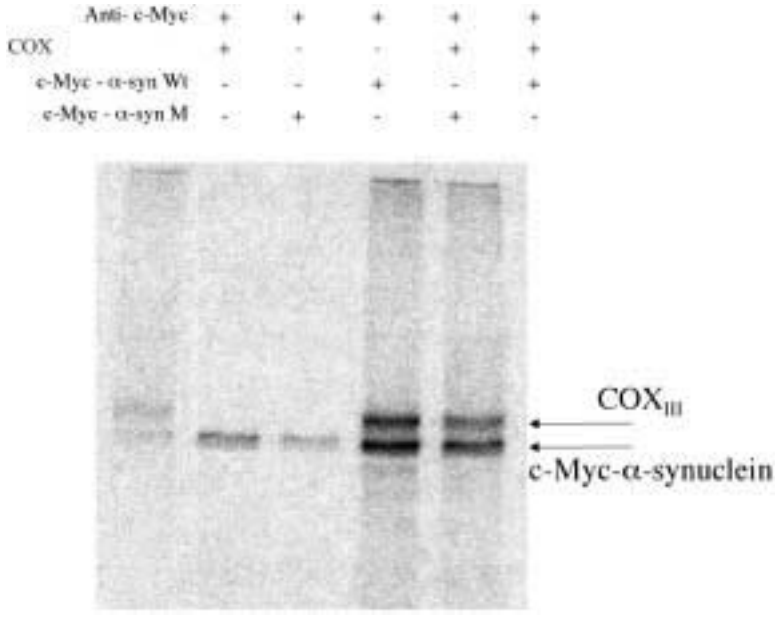

Fig. 3. Co-immunoprecipitation of wild-type and mutant (A53T) α-synuclein with COX_{III}. ^{35}S-Methyonine labeled α-synuclein (Myc fused) and COX_{III}, translated in vitro, were co-immunoprecipitated with monoclonal antibodies against c-Myc.

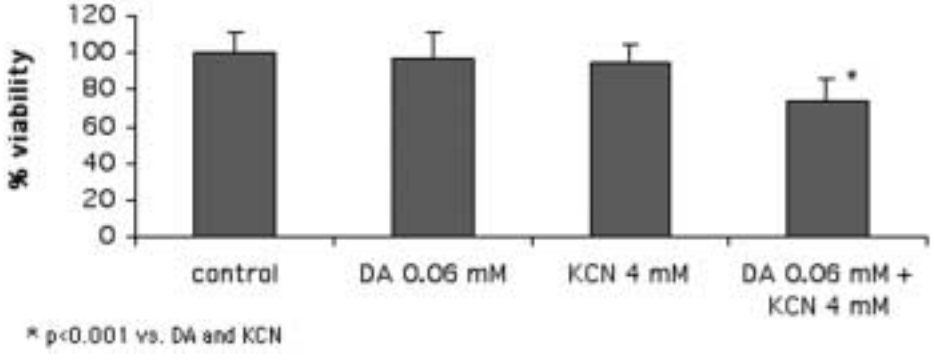

Fig. 4. Synergistic toxic effect of dopamine and potassium cyanide. SH-SY5Y neuroblastoma cells were treated with sublethal doses of dopamine (DA) and potassium cyanide (KCN), a complex IV inhibitor. Cell viability was indicated by Alamar blue (n = 4).

filament associated with ubiquitinated proteins. The mechanism leading to the formation of such abnormal deposits remain unclear. It was demonstrated that normal protein turnover is associated with covalent binding of ubiquitin to proteins, marking them for degradation by the ubiquitin-proteasome pathway. Failure of the ubiquitin-proteasome pathway to eliminate the ubiquitinated proteins due to oxidative stress, unrecognized substrate, mutations or impairment of proteasomal assembly caused protein accumulation (Alves-Rodrigues et al., 1998). It is unknown yet whether protein accumulation and aggregation in LB reflect initiation of deleterious process or attempt of protection by isolating the damaged proteins and neutralizing their possible toxic effects.

The aggregation of α-synuclein to itself or to other proteins is explained by its unique structure. The α-synuclein protein is composed of three modular domains, amino-terminal lipid binding α-helix, β-amyloid domain (including non-Aβ component, NAC) and a carboxy-terminal acidic tail (Davidson et al., 1998). In addition, α-synuclein exists as a mixture of rapidly equilibrating extended conformers, representing a class of "natively unfolded" proteins,

allowing protein-protein interactions and formation of α-helical structure, which favors binding to lipid membranes (Weinreb et al., 1996; Davidson et al., 1998). The relevance of α-synuclein aggregation to the LB structures was demonstrated by the accelerated aggregation in A53T and A30P mutated proteins (Narhi et al., 1999). Moreover, cells expressing the mutant form of α-synuclein show a decrease in the ubiquitin-proteasome activity (Tanaka et al., 2001).

Previous two-hybrid system studies with α-synuclein identified several proteins that are present in the LB or relevant to PD pathogenesis. Engelender et al. (1999) demonstrated that α-synuclein binds to synphilin-1, a 90 kD protein, which may act as an adaptor molecule anchoring other intracellular proteins. Another study revealed that α-synuclein interacts with Tat binding protein 1 (TBP1), a subunit of the PA700 regulatory complex of the 26S proteasome (Ghee et al., 2000). This finding may suggest that TBP1, a component of the protein degradation machinery, may be implicated with α-synuclein and might impair the proteasomal substrate recognition.

A recent report demonstrated the interaction of α-synuclein with presynaptic dopamine transporter accelerate cellular dopamine uptake and dopamine-induced cellular apoptosis (Lee et al., 2001). Additional immunoprecipitation experiments showed interactions of α-synuclein with the 14-3-3 protein, ERK and BAD, and suggested that it might act as a protein chaperone. It was also reported that accumulation of α-synuclein inhibits protein kinase C activity, which could contribute to cell death (Ostrerova et al., 1999).

Here we found that wild-type and mutant α-synuclein interacts with cytochrome C oxidase subunit III (COX_{III}), which is part of the complex IV of the mitochondrial respiratory chain. Complex IV receives electrons from cytochrome C and passes them to oxygen generating a proton and an ion gradient across the inner mitochondrial membrane. This gradient is used by ATP synthase (complex V) to drive ATP synthesis. COX consists of 13 subunits, the three largest (I, II and III) are encoded by mitochondrial genes, while the other 10 are encoded by the nucleus (Burke and Poyton, 1998).

The sensitivity of the dopaminergic cells to oxidative stress and mitochondrial dysfunction has been suggested to play a major role in the pathogenesis of PD (Mizuno et al., 1995). Moreover, postmortem studies in SN of PD patients demonstrated a significant decline in reduced glutathione and mitochondrial function (Sian et al., 1994; Schapira et al., 1990). The finding that MPTP, a parkinsonism-inducing compound, is a mitochondrial toxin that inhibits complex I of the respiratory chain, supports this theory of mitochondrial dysfunction. Furthermore, use of cell-lines repopulated with mitochondria from a PD patient (cybrid), significantly reduced the mitochondrial membrane potential and complex I and IV activities as compared with controls (Gu et al., 1998; Schapira et al., 1998; Trimmer et al., 2000). Indeed, overexpression of α-synuclein in GT1-7 cells causes the formation of α-synuclein immunopositive inclusion-like structures and mitochondrial alterations accompanied by increased levels of free radicals (Hsu et al., 2000). The importance of mitochondrial dysfunction in the pathophysiology of PD was also demonstrated in potassium cyanide (KCN) intoxication. KCN, a complex IV (COX) inhibitor taken during suicide attempts, caused the development of severe parkinsonian symptoms, including profound micrographia and hypersalivation (Feldman et al., 1984; Grandas et al., 1989; Rosenberg et al., 1989; Uitti et al., 1985). The importance of COX in PD pathology was further demonstrated in biopsies from a unique family with parkinsonism tendency that revealed 5–10% COX deficient muscle fibers (Casali et al., 2001). Thus, our finding that α-synuclein interacts with COX, the oxygen donor and a key enzyme of the respiratory chain and ATP production, supports the notion of mitochondrial involvement in PD. Complex IV inhibition, as well as complex I inhibition were shown to cause mitochondrial dysfunction and oxidative stress that induced degeneration of the dopaminergic cells. Moreover the COX interaction might also increase the leakage of mitochondrial cytochrome C, a known apoptotic initiator. Interestingly, cytochrome C, the COX ligand, was shown to co-localize with α-synuclein in LB of a PD patient (Hashimoto et al., 1999).

Dopaminergic neurons are exposed to massive oxidative stress in the SN as a result of high concentrations of iron, dopamine, and neuromelanin. The hypothesis that dopamine neurotoxicity might be involved in Parkinson's disease has been discussed extensively in our previous studies (Ziv et al., 1994, 1996; Offen et al., 1997, 1999; Barzilai et al., 2000) and in others publications (e.g., Simantov et al., 1996). Injection of dopamine into the striatum resulted in pre- and postsynaptic neuronal damage (Filloux and Townsend, 1993). Dopamine, by forming quinones and semiquinones and by its enzymatic oxidative metabolism by MAO, is also capable of attenuating the mitochondrial respiratory chain in dopaminergic neurons (Cohen et al., 1997).

Our experiment demonstrating a synergistic effect of COX inhibitor (KCN) and dopamine, suggests that a possible interaction of α-synuclein and COX in dopaminergic neurons in PD may be pathogenically important. It may enhance protein aggregation, leading to mitochondrial dysfunction and neuronal degeneration. Interestingly, such synergistic effect was shown when sublethal proteasome inhibitors were added to cells overexpressing wild type and mutant α-synuclein (Tanaka et al., 2001).

Present data suggest that oxidative stress, induced by dopamine metabolites, induces mitochondrial permeability, which enables COX interaction and aggregation with α-synuclein. Further studies of the binding site of α-synuclein and COX_{III}, may unmask the role of LB aggregation and mitochondrial failure in the pathophysiology of PD.

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