Mitochondrial Dysfunction in Skin Fibroblasts from a Parkinson’s Disease Patient with an alpha-Synuclein Triplication

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Abstract. Mitochondrial dysfunction has been frequently implicated in the neurodegenerative process that underlies Parkinson’s disease (PD), but the basis for this impairment is not fully understood. The goal of this study was to investigate the effects of α-synuclein (α-syn) gene multiplication on mitochondrial function in human tissue. To investigate this question, human fibroblasts were taken from a patient with parkinsonism carrying a triplication in the α-syn gene. Unexpectedly, the cells showed a significant decrease in cell growth compared to matched healthy controls. With regard to mitochondrial function, α-syn triplication fibroblasts exhibited a 39% decrease in ATP production, a 40% reduction in mitochondrial membrane potential, and a 49% reduction in complex I activity. Furthermore, they proved to be more sensitive to the effects of the nigrostrial toxicant paraquat compared to controls. Finally, siRNA knockdown of α-syn resulted in a partial rescue of mitochondrial impairment and reduction of paraquat-induced cell toxicity, suggesting that α-syn plays a causative role for mitochondrial dysfunction in these patient-derived peripheral skin fibroblasts.

Keywords: SNCA triplication, Parkinson’s disease, mitochondrial dysfunction, fibroblasts

INTRODUCTION

α-Synuclein (α-syn) is a major component of Lewy bodies, which are intraneuronal inclusions representing one of the hallmarks of Parkinson’s disease (PD). Although the function of α-syn is still unknown, an expanding body of literature indicates that high levels of α-syn are neurotoxic and lead to neurodegeneration. For example, overexpression of the α-syn gene (SNCA) in familial cases of PD due to SNCA duplications or triplications can lead to most if not all of the clinical and pathological features of PD [1, 2]. However, the mechanism by which this occurs is far from clear.

There is increasing evidence that α-syn can cause both mitochondrial dysfunction and increase in susceptibility to oxidative stress. α-Syn has been shown to be localized to the outer and inner mitochondrial membrane. It carries an N-terminal mitochondrial targeting signal which localizes α-syn to the inner mitochondrial membrane under physiological conditions, possibly mediated by the general import pore TOM 40 [3–5]. α-Syn over-expression causes an increase in reactive oxygen species, decrease in mitochondrial membrane potential, and impairment of cellular respiration in experimental models [4, 6–12]. In the basal ganglia of PD brains, in addition to Lewy bodies, α-syn accumulates within mitochondria and which also manifest as a decrease in complex I activity [4].

Given these experimental data, we decided to determine if similar findings could be replicated in human tissue from a 42 year old patient with a triplication of...
the SNCA gene, who has developed moderate parkinsonism over the last 5 years. This work was based on the rationale that finding abnormalities in both mitochondrial function and evidence of an increase in oxidative stress in fibroblasts from such a patient would add further credence to a close relationship between α-syn and mitochondrial dysfunction.

**MATERIALS AND METHODS**

**Skin biopsies of patient and control subjects**

One four mm skin punch biopsy was taken from our 42-year old patient with an SNCA triplication (SNCA-Tri) using a standard punch biopsy technique [13]. Single biopsies were also obtained from three healthy control individuals (46 yr female sibling of patient, 43 yr male, 35 yr female). All biopsies were taken from the upper inner arm, an area that is mostly unexposed to direct sunlight. The study and protocol had Institutional Review Board approval and all subjects gave written informed consent for this study. Detailed clinical information on the patient with the SNCA triplication is provided in Table 1.

**Fibroblast cell culture**

Primary fibroblasts from biopsies were derived from standard skin explant cultures and were banked at low passage numbers. Fibroblasts were cultured in Dulbecco’s minimum essential medium, high-glucose (DMEM) with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 200 mM glutamine, and 10 mM non-essential amino acids (all purchased from Invitrogen, Carlsbad, CA). Experiments were performed at passages 6–12.

**Cell viability and growth assay**

Cell viability was assessed using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Cat. No. G7890, Promega, Madison, WI), which is a fluorescent measure of lactate dehydrogenase (LDH) released from cells with a damaged membrane by conversion of resazurin into resorufin. The assay was performed in a 96-well format according to the manufacturers’ instructions. Briefly, 10,000 cells/well of control cells and 12,500 cells/well of SNCA-Tri fibroblasts were used for paraquat (PQ) exposure experiments. 12,500 cells/well were used in siRNA knockout experiment. Cell number was measured using WST-1 proliferation reagent (Roche, Indianapolis, IN) in a parallel plate.

The cell growth (5000 cells/well) was measured using WST-1 proliferation reagent. Cells were incubated for 1 hour at 37°C. All measurements were performed on a TECAN GENios (Switzerland). Rate of cell growth was calculated by the ratio of optical density over number of days during exponential growth.

**Measurement of mitochondrial membrane potential**

Fibroblasts were plated in 96-well plates (6,000 cells/well in the control and 7,500 cells/well in SNCA-Tri cells) were used for PQ exposure experiments (see below). After 24 hours, cells were changed to galactose medium and cultured for another 24 hours as described previously [14, 15]. The mitochondrial membrane potential was measured using the fluorescent dye tetramethylrhodamine methyl ester (TMRM) (Invitrogen) at the final concentration of 150 nM. Cells were incubated for 5 minutes at 37°C and then washed with phosphate buffer saline (PBS). To remove the plasma membrane contribution from the TMRM fluorescence, each assay was performed in parallel with 10 µM carbonyl cyanide 3-cholophenylhydrazone (CCCP) (Sigma, St Louis, MO), which collapses the mitochondrial membrane potential. All data were expressed as the total TMRM fluorescence minus the CCCP-treated TMRM fluorescence. Cell number was measured using WST-1 proliferation reagent in a parallel plate, according to the manufacturer’s instruction. All measurements were performed on a TECAN GENios.

**Assessment of mitochondrial complex I activity**

Complex I activity of the mitochondrial respiratory chain was assessed by Human Complex I Enzyme Activity Microplate Assay Kit (Cat. No. MS 141, Table 1 - Clinical information of subjects in this study

<table>
<thead>
<tr>
<th>Code</th>
<th>Patient ID</th>
<th>Gender</th>
<th>Age</th>
<th>Mutation</th>
<th>Clinical Description</th>
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<tr>
<td>Con 1</td>
<td>1604</td>
<td>Female</td>
<td>35</td>
<td>None</td>
<td>Healthy control</td>
</tr>
<tr>
<td>Con 2</td>
<td>1761</td>
<td>Female</td>
<td>46</td>
<td>None</td>
<td>Healthy sibling control</td>
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<tr>
<td>Con 3</td>
<td>1827</td>
<td>Male</td>
<td>43</td>
<td>None</td>
<td>Healthy control</td>
</tr>
<tr>
<td>SNCA-Tri</td>
<td>1754</td>
<td>Male</td>
<td>42</td>
<td>SNCA triplication</td>
<td>See case description</td>
</tr>
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</table>
Mitoscience Inc, Eugene, OR) to determine the activity of mitochondrial OXPHOS complex I, according to the manufacturer’s instruction. All measurements were performed on the fluorescent plate reader TECAN GENios.

Adenosine triphosphate synthesis

Cellular adenosine triphosphate (ATP) levels were measured using the ATPlite kit (Cat. No. 6016943, Perkin Elmer, Waltham, MA) based on the reaction of ATP with added luciferase and D-luciferin, according to the manufacturer’s instruction. ATP synthesis assays were performed on digitonin-treated fibroblasts [16]. Briefly, for PQ exposure (see below), cells were plated (6,000 cells/well in control cells and 7,500 cells/well in SNCA-Tri cells) for 24 hours prior to exposure to PQ.

Toxin exposure

Cells were exposed to freshly prepared PQ (N,N′-dimethyl-4,4′-bipyridinium dichloride) (Sigma, M2254) at 300 μM final concentration in culture media for 48 hours without media change prior to analysis. According to a dose-response test of control fibroblasts with PQ, 300 μM of PQ showed about 25% cell death at 48 hrs.

Small interfering RNA-mediated knockdown

The sequence of SNCA siRNA were used after the method of Fountaine et al. 2008 [17]. Custom siRNA (Dharmacon, Lafayette, CO) at a concentration of 75 nM (scrambled or α-syn targeted siRNA, SNCA1) was transfected at 90% cell confluency using 0.5 mM Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instruction. Transfection efficiency was tested to be >90% with siGLO Cyclophilin B control siRNA (Dharmacon) in control fibroblasts.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from fibroblasts using TRIzol® (Invitrogen). cDNA from 0.5 μg total RNA was synthesized by reverse transcriptase (iScript cDNA synthesis kit, BioRad, Hercules, CA). Primer sequences of SNCA are SNCA_F: AGTTGTTGCG TGTGCTTAG and SNCA_R: CTCCTCCTTGGT TTTGGAG and of β-actin are Hu Actin_F: CAGC AGATGGTGGATCAAGAAG and Hu Actin_R: CACATT TGCGGTGAGCAT. The PCR products of SNCA and β-actin (as internal control) were amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Amplification of the PCR products was quantitatively measured by the ABI 7000 Sequence Detection System (Applied Biosystems). 2−ΔΔCt method was used in all quantitative PCR analyses.

Statistical analysis

Data analysis was conducted on three or more biological replicates. Differences among means were analyzed using student’s t-test and one-way ANOVA. Newman-Keuls post-hoc analysis was used when differences were observed in ANOVA testing (p < 0.05).

RESULTS

SNCA triplication fibroblasts under naïve growth conditions

Fibroblast cultures of our patient with an SNCA triplication (SNCA-Tri) demonstrated a 24% decrease in cell proliferation rate compared to matched healthy control subjects (Con) (Fig. 1A). Owing to the slow growth rate of the SNCA-Tri fibroblasts, we investigated whether or not mitochondrial function would be impacted. We tested complex I activity because it has been consistently described as being impaired in familial and sporadic PD. Kinetic assessment of the activity of complex I in cell extracts showed that complex I activity was 49% lower in the SNCA-Tri cells compared to controls (Fig. 1B). Activities of other mitochondrial complexes were not measured in this study.

The complex I deficiency was associated with impaired mitochondrial ATP production. ATP synthesis was diminished by 39% after specific substrates (pyruvate and malate) for complex I (Fig. 1C) in the SNCA-Tri fibroblasts. We attribute these finding to impairment of mitochondrial function and not to a decrease in number of mitochondria, since the ratio of mitochondrial DNA/nuclear DNA did not show a significant difference in the control and patient specific cultures (Fig. 1D). In summary, the patient-derived SNCA triplication fibroblasts demonstrated a pronounced impairment of mitochondrial function under naïve culture conditions.

Increased susceptibility to oxidative stress of SNCA-Tri fibroblasts after paraquat exposure

Given the relationship between mitochondrial dysfunction and oxidative stress, we hypothesized that the...
patient SNCA-Tri fibroblasts would be more susceptible to oxidative stress compared to control fibroblasts. In these experiments, we exposed the cells to 300 μM of the herbicide PQ for 48 hrs. We tested cell viability and cell membrane damage using lactate dehydrogenase (LDH) release. Under naïve conditions, the SNCA-Tri fibroblasts already showed a slight increase of 33% in LDH release compared to controls 24 hrs after plating. When the cells were treated with PQ, cell viability in SNCA-Tri fibroblasts was greatly affected. Cellular LDH release showed 46% increase after PQ treatment in cells from the SNCA-Tri carrier compared to controls (Fig. 2A). In control fibroblasts compared to SNCA-Tri cells, significant reduction of mitochondrial membrane potential and cellular ATP were observed by 40% and 47% under naïve conditions and by 51% and 59% after PQ exposure, respectively (Fig. 2B and C).

We also compared the difference (in %) in untreated and PQ exposed fibroblasts in the control group and the SNCA-Tri fibroblasts (Fig. 2D). The change difference for LDH release in controls was 27% after PQ treatment, whereas the SNCA-Tri fibroblasts showed a 42% increase in LDH release (1.6 fold change). Cellular ATP production was decreased upon PQ treatment in controls by 33% compared to 56% in SNCA-Tri fibroblasts (1.7 fold change). Membrane potential was decreased by 40% in controls upon PQ treatment and 61% in SNCA-Tri fibroblasts (1.5 fold change). These comparisons show that the SNCA-Tri fibroblasts are more vulnerable to oxidative stress than the matched mutation-negative healthy control cells.

Finally, we measured SNCA transcription levels after PQ exposure. We observed a 2.6-fold increase of α-syn mRNA in the control fibroblasts, which is well described in cell and animal models of PD, but in the SNCA-Tri fibroblasts, SNCA upregulation was unexpectedly high with a 7.5 fold increase in the SNCA-Tri cells compared to naïve expression levels, which is 3.2 fold increase of mRNA transcript in SNCA-Tri fibroblasts over control fibroblasts after PQ treatment (Fig. 2E).
Fig. 2. SNCA-Tri fibroblasts treated with PQ. (A) Under naive conditions, SNCA-Tri fibroblasts showed a 33% increase in LDH release (*p<0.0008), after PQ exposure, an increase of LDH release (46%, **p<0.0007) showed reduced cell viability in the patient fibroblasts compared to controls. (B) Mitochondrial membrane potential was reduced by 40% under naive conditions (*p<0.0001) and further impaired by 51% (***p<0.0001) in SNCA-Tri carriers compared to controls after PQ exposure. (C) Cellular ATP levels under naive conditions showed a 47% reduction (*p<0.0001); after PQ exposure, a 59% decrease of cellular ATP between controls and SNCA-Tri were detected (****p<0.0001). (D) Comparison of % change in untreated and PQ exposed controls or SNCA-Tri. For LDH release, we measured a 27% change in controls, and a 42% change in SNCA-Tri, thus 1.6 fold difference (*p<0.0084); Cellular ATP levels showed a 33% and 56% difference in controls and SNCA-Tri, respectively, a 1.7 fold change (**p<0.0032); mitochondrial membrane potential showed a 40% and 61% decrease in controls and SNCA-Tri, respectively leading to a 1.5 fold change (***p<0.0011). (E) After PQ exposure, the level of a-syn mRNA expression increased by 2.6 fold (**p<0.0012) in controls and 7.5 fold (****p<0.0006) in SNCA-Tri, which equaled a 3.2 fold difference (p<0.0039) in SNCA-Tri culture compared to control cultures. Data are presented as mean ± standard error of the mean (SEM) compared to the controls (n=9 in the controls; n=9 in the SNCA-Tri). p-value of each study was determined by student’s unpaired t-test.
Mitochondrial malfunction can be partially reversed by RNAi knockdown of α-syn

Since our working hypothesis was that these effects are due to the gene multiplication of the SNCA gene, we tested if downregulation of α-syn would ameliorate the observed changes in cell viability, mitochondrial function, and susceptibility to oxidative stress in fibroblasts from our SNCA triplication patient. PQ-exposed SNCA-Tri fibroblasts were transfected with small interfering RNA (siRNA) against SNCA mRNA, transfection reagent Lipofectamine™2000 only, or scrambled siRNA, and harvested 48 hours after transfection. α-Syn mRNA expression in SNCA-Tri fibroblasts was reduced by approximately 76% 24 hours after siRNA transfection compared to Lipofectamine™2000 alone or scrambled siRNA (Fig. 3A). There was no significant difference between mitochondrial function under control conditions and scrambled siRNA or Lipofectamine™2000 alone. In the PQ-treated SNCA-Tri culture transfected with siRNA against α-syn after 48 hours, a partial but significant restoration of cell damage measured by LDH release by 31% (Fig. 3B), the increase in cellular ATP production by 37% (Fig. 3C), and mitochondrial membrane potential by 36% (Fig. 3D). This clearly shows that suppression of α-syn partially reverses mitochondrial malfunction after a neurotoxin insult causatively linking α-syn overexpression due to the SNCA triplication to mitochondrial impairment.

DISCUSSION

This is the first report linking mitochondrial dysfunction and SNCA multiplication in human fibroblasts. We evaluated skin fibroblasts from a PD-affected carrier of the Iowa kindred [1, 19], a family with autosomal-dominant early-onset parkinsonism caused by a triplication in the SNCA gene.

Fibroblasts of SNCA triplication carrier shows dramatic reduction in mitochondrial complex I activity

The link between mitochondrial dysfunction and sporadic PD was first shown as a specific reduction of complex I in the substantia nigra (SN) of patients.
Gene-environment interaction: SNCA triplication
human fibroblasts show higher vulnerability after paraquat exposure

In order to investigate susceptibility to oxidative stress in the SNCA triplication fibroblasts, we applied the neurotoxin and herbicide PQ which has been implicated as a PD risk factor in epidemiological studies and induces nigrostriatal damage in laboratory animal models [30–32]. Interestingly, one recent study suggested both SNCA promoter variants and PQ exposure may have an impact on the risk for developing PD [33]. PQ has been evident to trigger up-regulation of α-syn in neuroblastoma cells and in mice [34, 35] and induces oxidative stress by increased production of reactive oxygen species through redox cycling [36].

We exposed fibroblasts to 300 μM PQ did measurements after 48 hours and detected an increase of LDH release indicative of cellular damage. This increase was significantly higher in SNCA-Tri fibroblasts and as compared to healthy controls. When assaying mitochondrial function, we also observed a significant decrease in membrane potential and ATP levels in SNCA-Tri compared to controls. While the SNCA triplication itself is already affecting the viability and mitochondrial function of the cells under naive culture conditions, the additional oxidative stress caused by PQ exposure is exacerbating the mitochondrial dysfunction in a way that is significantly enhanced over that seen in fibroblasts from normal subjects. This provides an interesting and potentially useful model of a gene-environment interaction that could shed light on the epidemiological data investigating SNCA gene variants and pesticide exposure.

Mitochondrial function and cellular damage was partially “rescued” after siRNA knockdown of α-syn in fibroblasts after PQ treatment. We observed a significant increase in membrane potential and cellular ATP synthesis as well as a decrease in LDH release supporting the hypothesis that α-syn expression levels are directly related to mitochondrial dysfunction.

In summary, we have shown for the first time mitochondrial impairment in patient-derived fibroblasts from a patient with an SNCA triplication which can be partially rescued by the knockdown of α-syn. Additional studies of patient-derived differentiated dopaminergic neurons, a tissue of high-energy expenditure in the resting state, will be an important future step to determine if these effects are more pronounced, thus making the brain and specifically dopamine neurons more vulnerable.

**SUPPLEMENTARY DATA**

**CLINICAL CASE DESCRIPTION OF SNCA-TRIP (PI-1754)**

The patient was a 42-year-old at the time of the skin biopsy. His initial symptoms began at age 41, which were fatigue, resting tremor and decreased dexterity. An MRI scan was unremarkable. He exhibited a resting and mild intention tremor, but his gait was normal. Thus, he appeared to have mild Parkinson’s disease, but his symptoms were mild and no medication was required.

At the time of the clinical examination in summer 2008, he noted mild problems with recent memory. He
did not experience any psychiatric symptoms such as delusions or hallucinations but feels somewhat anxious and slightly depressed. From time to time he had noted periodic blurring of vision and diplopia. He developed motor fluctuations less than a year after initiation of a dopamine therapy. When he is in the “off” state or when the medication wears out, his speech slows, he drools, his handwriting worsens, and he has more difficulty with dexterity such as dressing or hygienic activities.

Modified Hoehn and Yahr staging
The patient has bilateral disease with recovery on pull test, which is equivalent with Hoehn and Yahr Stage 2.5.

Unified Parkinson disease rating scale (UPDRS, parts I-III)
At age 42 were 8 points for part I, non-motor aspects of experiences of daily living, 15 points for part II, motor aspects of experiences of daily living, and 25 points for part III, motor examination.

Cognition
One year after onset of illness, he underwent a mini mental state examination (MMSE) and scored normal 30/30. Three years after onset of symptoms at age 44, he scored 25/30 using the Montreal cognitive assessment (MOCA), which indicates a mild cognitive decline.

Autonomic symptoms
He has mild constipation and bladder urgency and his voice is weak. He is experiencing urinary urgency and occasional constipation.

Sleep
Although we did not perform a formal assessment, he exhibits vivid dreams and is ‘trashing around’ during his sleep consistent with REM Behavior Disorder (RBD). His Epworth sleepiness scale to determine the level of daytime sleepiness is 9/24 points which is within the high normal range of 0–10.

Olfactory function
The patient scored 6/12 points on the brief smell identification test (Brief-Smell identification test (B-SIT) Sensonics) at age 42, which scores below the 1% tile relative to matched age and gender group. Offactory function of the patient was tested again at age 45 using the 40-item University of Pennsylvania Smell Identification Test (UPSIT). He scored 11/40. On an absolute scale, this represents total anosmia. He fell below the 5% percentile range (5th percentile being a score of 27/40 for his age group).

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