Curcumin protects against A53T alpha-synuclein-induced toxicity in a PC12 inducible cell model for Parkinsonism

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A B S T R A C T

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies. The pathogenesis of PD remains incompletely understood, but it appears to involve both genetic susceptibility and environmental factors. Treatment for PD that prevents neuronal death in the dopaminergic system and abnormal protein deposition in the brain is not yet available. Evidence from human and animal studies has suggested that oxidative damage critically contributes to neuronal loss in PD. Here we test whether curcumin, a potent antioxidant compound, derived from the curry spice turmeric, can protect against mutant A53T α-synuclein-induced cell death. We used PC12 cells that inducibly express A53T α-synuclein. We found that curcumin protected against A53T α-synuclein-induced cell death in a dose-dependent manner. We further found that curcumin can reduce mutant α-synuclein-induced intracellular reactive oxygen species (ROS) levels, mitochondrial depolarization, cytochrome c release, and caspase-9 and caspase-3 activation. This study demonstrate that curcumin protected against A53T mutant α-synuclein-induced cell death via inhibition of oxidative stress and the mitochondrial cell death pathway, suggesting that curcumin may be a candidate neuroprotective agent for A53T α-synuclein-linked Parkinsonism, and possibly for other genetic or sporadic forms of PD.

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1. Introduction

Parkinson's disease (PD) is a neurological disorder associated with tremor, muscle rigidity, disturbances of balance, and bradykinesia. Two pathological hallmarks of PD are selective loss of dopaminergic neurons and accumulation of α-synuclein and other proteins in Lewy bodies [1–4]. Although dopamine replacement can alleviate symptoms of the disorder, there is no proven therapy to halt the underlying progressive degeneration of dopamine-containing neurons in the brain [1,5].

α-Synuclein was the first genetically identified PD-associated protein [2]. α-Synuclein (A30P, A53T and E46K) mutations cause rare familial PD [6–8]. Genetic duplication or triplication at the α-synuclein locus leading to over-expression of α-synuclein protein also cause genetic PD [9]. α-Synuclein is the major constituent of the Lewy bodies [6–8]. Variation in levels of α-synuclein expression inherited by promoter variants may also contribute to the risk of developing PD [10,11]. The function of α-synuclein is not well understood. Recent studies suggest that α-synuclein may play a role in neuronal plasticity and neurotransmission [12,13] and may interact with phospholipid membranes and regulate synaptic vesicle dynamics [14,15]. Transgenic mice and flies expressing human wild type or mutant α-synuclein exhibit neuronal dysfunction, degeneration and abnormal cellular accumulation of α-synuclein [16–20]. In vitro, we and others found that expression of A53T α-synuclein increased intracellular reactive oxygen species (ROS) levels, mitochondrial dysfunction and caused cell death [21–32]. Previously, we generated an A53T mutant α-synuclein inducible PC12 cell line using the Tet-Off gene expression regulatory system [21]. Inducible expression of A53T α-synuclein alone causes cellular toxicity. In this study, we employed this cell model to test....

Abbreviations: PD, Parkinson's disease; ROS, reactive oxygen species; NGF, nerve growth factor; DCFDA, 2,7'-dichlorofluorescein diacetate; FBS, fetal bovine serum; Dox, doxycycline; LDH, Lactate dehydrogenase; JC-1, 5,5'-dichloro-1,1'-tetraethylbenzimidazolylcarbocyanine iodide.

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the neuroprotective effect of curcumin and to explore the potential protective mechanisms.

Curcumin is a component of yellow curry spice derived from turmeric which is used as a food preservative and herbal medicine in India [33]. Curcumin is an antioxidant and anti-inflammatory compound. In vitro studies show that curcumin has anti-amyloid, cholesterol-lowering and hemostatic properties. Studies in animal models of Alzheimer’s disease (AD) indicate that curcumin can decrease the amyloid pathology of AD. In this study, we tested the hypothesis that curcumin protects against mutant A53T α-synuclein-induced cell death using PC12 cells inducibly expressing A53T α-synuclein. We found that curcumin protected against A53T α-synuclein-induced cell death in a dose-dependent manner. Curcumin reduced mutant α-synuclein-induced intracellular reactive oxygen species (ROS) levels, mitochondrial depolarization, cytochrome c release, and caspase-9 and caspase-3 activation. These results indicate that curcumin protected against A53T α-synuclein-induced cell death via inhibition of oxidative stress and mitochondrial cell death pathway, suggesting that curcumin may be a candidate neuroprotective agent for A53T-linked PD intervention.

2. Materials and methods

2.1. Materials

Media and N2 supplements for cell culture were from Invitrogen (Carlsbad, CA, USA). Nerve growth factor (NGF) was from Roche (Indianapolis, IN, USA), and 2',7'-dichlorofluorescein diacetate (DCFDA) from Molecular Probes (Eugene, OR, USA). Anti-α-synuclein monoclonal antibody (directed against amino acids 15–123 of rat α-synuclein but exhibiting good reaction with human-α-synuclein) was from BD Biosciences (Palo Alto, CA, USA).

2.2. Cell culture, inducible cell lines, cell death assays

PC12 cell lines expressing inducible A53T α-synuclein were described previously [21,34], and were grown in non-induction maintaining medium: DMEM containing 10% horse serum, 5% Fetal bovine serum (FBS), 100 μg/ml G418, 200 μg/ml hygromycin B (Clontech) and 200 ng/ml doxycycline (Dox). Anti-α-synuclein monoclonal antibody (directed against mouse α-synuclein but exhibiting good reaction with human-α-synuclein) was from BD Biosciences (Roche, Indianapolis, IN).

2.3. Western blot analysis and antibody

Cells were harvested in lysis buffer [20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 5 mM NaF]. Lysates were resolved on 4–12% NuPAGE Bis–Tris gels (30 μg/lane) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) as described previously [21]. The membranes were blocked in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and then probed with anti-α-synuclein monoclonal antibodies. Proteins were detected using enhanced chemiluminescence reagents (NEN Life Science, Boston, MA, USA).

2.4. Measurement of intracellular ROS

The levels of intracellular ROS were measured by DCFDA (molecular probes) as previously described [21]. Briefly, cells were washed with PBS, then incubated for 45 min with DCFDA, which is initially non-fluorescent and is converted by oxidation to the fluorescent molecular 2,7-dichlorofluorescein (DCF). DCF was then quantified using a CytoFluor Multi-well Plate Reader, Series 400 (Perceptive Biosystems, USA) with 485 nm excitation and 538 nm emission filters.

2.5. Measurement of cellular caspase activity

Cells were harvested in cell lysis buffer (50 mM HEPES, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS and 0.1% Triton X-100). DEVD-p-nitroanilide and LEHD-p-nitroanilide were the substrates for caspase-3, and caspase-9, respectively. The experiments were performed according to the manufacturer’s protocol (Biosource International).

2.6. Mitochondrial membrane potential and cytochrome c assay

Loss of mitochondrial membrane potential, indicative of apoptosis, was detected using JC-1 MitoPT™ detection kit (B-bridge International Inc.) according to the manufacturer’s protocol. Briefly, cells were incubated with MitoPT dye (JC-1) at 37 °C for 15 min in a CO2 incubator. Fifty thousand cells from each experiment group were dispensed in 100 μl assay buffer in a black flat-bottom 96-well plate. The red JC-1 fluorescence was measured using a CytoFluor Multi-well Plate Reader, Series 400 (Perceptive Biosystems) with 485 nm excitation and 590 nm emission filters. By comparing the average of red fluorescent signal in induced versus non-induced control samples, the loss of mitochondrial membrane potential can be monitored. The apoptotic cells generate a lower reading of red fluorescence. For cytochrome c ELISA, mitochondrial and cytoplasmic fractions were prepared using the Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA, USA). Cytochrome c levels were measured using a commercially available ELISA kit (R&D Systems).

2.7. Data analysis

Quantitative data are expressed as arithmetic means ± S.E. based on at least three separate experiments. The difference among experimental groups was statistically analyzed by Student’s t-test or an analysis of variance (ANOVA). A p value <0.05 was considered significant.

3. Results

3.1. Curcumin attenuates A53T-α-synuclein-induced cell death

To study the effect of curcumin (Fig. 1A), we employed the rat pheochromocytoma cell line PC12, that inducibly expresses PD-linked mutant A53T α-synuclein using the Tet-off gene regulatory system [21]. Consistent with previous findings, inducible expression of mutant A53T α-synuclein increased cell death about 2–3 fold after 6 days differentiation and induction of expression, when compared with the non-induced condition (Fig. 1B and C). We treated with curcumin at the same time as we induced A53T α-synuclein expression. Curcumin treatment significantly attenuated cell death in a concentration-dependent manner (Fig. 1B and C). 500 nM curcumin reached its maximal protective effect against A53T α-synuclein toxicity. The EC50 was approximately 200 nM.
Curcumin did not alter the survival of cells not expressing mutant A53T α-synuclein. Curcumin treatment also had no effect on A53T α-synuclein expression levels (Fig. 1D).

3.2. Curcumin reduced A53T-α-synuclein-induced intracellular ROS

To further study the protective mechanism of curcumin, we measured intracellular ROS using the redox-sensitive fluorophore DCFDA. Non-fluorescent DCFDA is converted by oxidation to the fluorescent molecule DCF. We found that the level of DCF in PC12 cells inducibly expressing A53T α-synuclein increased on the first day of induction, and was more than three-fold higher than non-expressing control cells on the 6th day of induction (Fig. 2). The increase in DCF fluorescence was significantly reversed by 500 nM curcumin.

3.3. Curcumin partially reversed A53T-α-synuclein-induced mitochondrial depolarization and cytochrome c release

Mitochondrial dysfunction has been implicated in the pathogenesis of PD. Previously we and others have shown that mutations of α-synuclein induced mitochondrial depolarization [21,34–37]. To test whether curcumin alters A53T α-synuclein-induced...
Curcumin reduced A53T-α-synuclein-induced mitochondrial dysfunction.

Release of cytochrome c into the cytosol is a marker of mitochondrial dysfunction after mitochondrial depolarization. To further assess whether curcumin alters A53T-induced mitochondrial dysfunction, we measured cytochrome c in the cytosol using an ELISA. Induction of A53T expression increased the release of cytochrome c from the mitochondria and its accumulation in the cytosol (Fig. 4). Treatment with 500 nM curcumin significantly reduced cytochrome c release. Data are shown as the mean ± SE for three separate experiments. *p < 0.05 vs cells without doxycycline and curcumin treatment by ANOVA.

4. Discussion

In this study, we investigated the effects of curcumin on PD-linked mutant A53T α-synuclein toxicity using a tet-off cell model of familial Parkinsonism. Curcumin attenuated A53T α-synuclein-induced cell death via reducing the intracellular ROS level, mitochondrial depolarization, cytochrome c release, and caspase-3 and caspase-9 activation. This is the first study to use a PC12 inducible cell model showing that curcumin can protect against A53T mutant α-synuclein-induced cell death, suggesting that curcumin may be a potential neuroprotective agent for A53T α-synuclein-linked PD. Since α-synuclein has been linked to familial and sporadic PD, curcumin may be a candidate for experimental therapeutics of PD.

Curcumin is an antioxidant and anti-inflammatory compound, and is several times more potent than vitamin E as a free radical scavenger [38], protecting the brain from lipid peroxidation [39], and scavenging NO-based radicals [40]. Treatment with doses of curcumin of 160–5000 ppm reduces oxidative damage and amyloid pathology in an AD transgenic mouse [41]. Curcumin can penetrate the blood brain barrier, is relatively non-toxic, and has few side effects at high doses [41,42]. Because of its use as a food additive and its potential for cancer chemoprevention, curcumin has undergone extensive toxicological screening and pre-clinical investigation in rats, mice, dogs, and monkeys [43]. In clinical trials, patients have not shown adverse effects with doses from 2000 to 8000 mg/day [44,45]. These studies indicate that curcumin is relatively safe for animals and humans and is a potentially attractive therapeutic candidate. Previous reports show that curcumin reduces 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (OHDA) toxicity [46–48]. Here, our results showed curcumin protected against mutant α-synuclein-induced cell death via reducing oxidative damage and blocking the mitochondrial cell death pathway, in consistent with a recent study using a SH-SY5Y transient transfection cell model [49]. A caveat to...
curcumin in vivo studies is that it requires large doses to achieve the neuroprotective effect since curcumin is poorly absorbed and heavily metabolized. Development of curcumin derivatives with high bioavailability may lead to the wide therapeutic utility of curcumin for PD intervention.

Expression of α-synuclein has been achieved in a number of cellular systems, with results ranging from a lack of any effect after overexpression, to adverse effects of varying severity [25,27,29–32,23,24,26,28]. Recently we have found that cell toxicity was directly caused by expression of A53T mutant α-synuclein without additional stressor stimuli in low serum (1% HS and 0.5% FBS) serum media containing N2 and NFG [21]. In this cell model, inducing expression of A53T α-synuclein in differentiated PC12 cells decreases proteasome activity and increases the intracellular ROS level leading to both ER stress and mitochondrial dysfunction and causes up to ∼40% cell death [21]. Moreover oxidative stressors can strikingly enhance A53T α-synuclein-induced death in this model [22]. In this study, we found that curcumin significantly reduced mutant α-synuclein-induced ROS level and attenuated cell death. Oxidative stress is believed to be a common mechanism underlying the pathogenesis of age-related neurodegenerative diseases [50,51]. Ageing cells and organisms accumulate increased levels of oxidant-damaged DNA, lipid and protein, and have reduced levels of oxidant scavenger enzymes and activities [52]. The increase of intracellular oxidant levels has two potentially harmful effects: damage to cellular components and activation of specific stress-signaling pathways, which can lead to cell death. Both of these effects can also influence numerous cellular processes linked to ageing and the development of age-related diseases including PD [52]. A variety of markers and indices in PD patients and animal models indicate involvement of oxygen-free radicals and oxidative stress in the pathogenesis of PD, including lipid, protein and DNA oxidation [53–55], reduction of gluthathione [54] and mitochondrial complex I activity as well as an increase of iron and reduction of ferritin concentrations in PD patients’ brains [56,57]. Moreover, levels of two free radical scavengers, superoxide dismutase and glutathione, are altered in the nigra of PD brains [54]. The enzymatic metabolism of dopamine generates H2O2 and dopamine autooxidation forms semiquinones, which allows for the generation of ROS [58–60]. α-Synuclein can enhance the formation of H2O2 in vitro [61,62]. Intracellular overexpression of α-synuclein generates excess ROS and causes oxidative stress to the cells, leading to disruption in redox homeostasis cell metabolism, free radical generation, lipid peroxidation, cholesterol and protein oxidation. Our results indicate that the antioxidant effect of curcumin as a scavenger of ROS is an early step and major pharmacological mechanism in protecting against mutant α-synuclein-induced cell death.

In conclusion, we demonstrated that curcumin can protect against mutant A53T α-synuclein-induced cell death via reducing ROS, mitochondrial depolarization, cytochrome c release, and caspase-9 and caspase-3 activation. The tet-off PC12 inducible α-synuclein cell model may provide a valuable system for preclinical investigation of PD therapeutics. Our results suggest that curcumin may be a promising attractive therapeutic compound for PD.

References


