Curcumin Accelerates the Clearance of Mutant α-synuclein and Injured Mitochondria *via* Autophagy Induction

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Background

It has been demonstrated that misfolding and aggregation of proteins, such as mutant α-synucleins (A53T, A30P, E46k) and α-synuclein multiplications (Gorman, 2008; Yu and Lyubchenko, 2009), as well as neurotoxins-related mitochondrial dysfunction (Sherer et al., 2003) are important in the pathogenesis of Parkinson's disease (PD) . Recently, defective degradation of the abnormal proteins has emerged as the leading mechanism of cell death in PD and other neurodegenerative disorders (Pan et al., 2008; Rami, 2009). Autophagy is involved not only in clearing misfolded proteins but also in injured mitochondria (Rubinsztein, 2006). Inhibition of autophagy may caused the increase of mitochondrial load such as mitochondrial complex IV and cytochrome *c* (Sarker et al. 2007), or cause the delay in the clearance of misfolded proteins (Hara et al., 2006). We have recently reported that compounds clearing misfolded proteins or injured mitochondrial may be protective for PD (Pan et al., 2009; Wu et al., 2010).

Curcumin (1,7-bis[4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione), is a widely studied natural phenolic compound with a variety of biological activities. Recently, the anti-apoptotic effects of curcumin have been reported (Chen et al., 2006) and the neuroprotective properties of curcumin have also been demonstrated in the toxin model of PD (Zbarsky et al., 2005). Additionally, it has been reported that curcumin has inhibitory effects on the aggregation of α-synuclein (Pandey et al., 2008). Recent reports that curcumin induces autophagy in malignant glioma cells (Aoki et al., 2007) led us to hypothesize that curcumin may have beneficial effects on PD *via* enhanced clearance of injured mitochondrial and/or of α-synuclein through autophagy induction.

In this study, we explored the role of curcumin in the induction of autophagy and determined neuroprotective effects of curcumin on mitochondrial complex I inhibitor 1-methyl-4-phenylpridinium ions (MPP+)-induced apoptosis and on the degradation of α-synuclein.

Methods

Stable inducible PC12 cell lines expressing HA-tagged A30P, A53T mutant or wild-type α-synuclein respectively (kind gift of professor David Rubinsztein from Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge), were maintained at Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% fetal bovine serum, 75 μg/ml hygromycin B, and 100 μg/ml G418 at 37°C, 10% CO2. The expression of transgenes including A30P, A53T mutant or wild-type α-synuclein were induced by doxycycline (1 μg/ml) for 48 hours. Doxycycline-uninduced PC12 cells were transfected with autophagy-related gene Beclin 1 siRNA for 48 h followed by addition with curcumin. Doxycycline-uninduced PC12 cells were differentiated with NGF (100 ng/ml) for 5 days followed by exposure to mitochondrial complex I inhibitor MPP+ (0.5mM) for 24 h with or without curcumin pretreatment.

Immunoblotting assay were used to detect protein levels using specific antibodies. ELISA assay was used to verify the occurrence of apoptosis. The protein level of LC3-II was used to detect the induction of autophagy. The mitochondrial load was evaluated by the levels of mitochondrial proteins including mitochondrial complex IV and cytochrome c. The protein levels of α-synuclein was determined by immunoblotting assay with anti-HA antibody.

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Figures

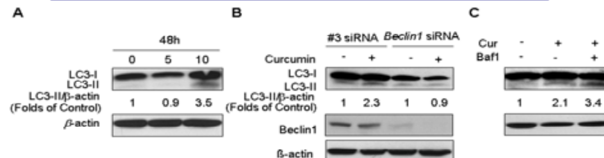

Fig. 1 Induction of autophagy by curcumin. The un-induced stable inducible PC12 cells expressing A53T mutant α-synuclein were treated with curcumin at 5 μM or 10 μM for 48 h (A). Four hours before harvest, cells were treated with (+) or without (-) 200 nM of bafilomycin A1 (Baf1), an autophagosome-lysosome fusion blocker (B). Or the cells were transfected with *Beclin1* siRNA or scrambled control #3 siRNA for 48 h followed by treatment with (+) or without (-) 10 μM curcumin for another 48h (C). Total proteins were isolated and subjected to immunoblotting assay using anti-LC3 antibody. The relative density of LC3 to β-actin was quantified by densitometric analysis.

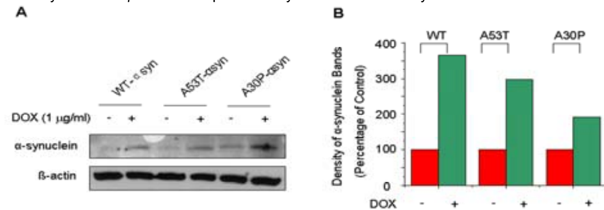

Fig. 2 Transgene expression of α-synuclein in PC12 cell lines. Stable inducible PC12 cell lines expressing HA-tagged A30P, A53T mutant and wild-type α-synuclein were induced with (+) or without (-) doxycycline (1μg/ml) for 48 h. The expression of transgene was switched off for 24 h. Total proteins were isolated and subjected to immunoblotting assay and the expression of α-synuclein in different cell lines were determined using anti-HA antibody (A). Densitometric analysis was applied to quantify the protein level of α-synuclein (B). DOX = Doxycycline

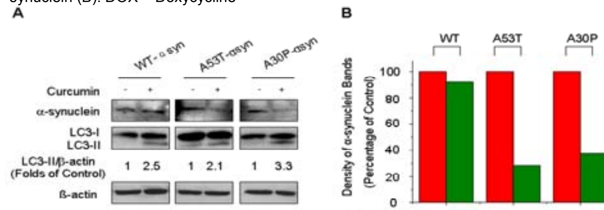

Fig. 3 Clearance of α-synuclein by curcumin. Stable inducible PC12 cell lines expressing HA-tagged A30P, A53T mutant and wild-type α-synuclein were induced with doxycycline (1μg/ml) for 48 h. Then the cultures were changed with fresh medium containing with (+) or without (-) curcumin (10 uM) for another 48 h. Total proteins were isolated and subjected to immunoblotting assay. The expression of α-synuclein was determined using anti-HA antibody (A). The induction of autophagy was evaluated by immunoblotting assay using anti-LC3 antibody (A). The protein level of α-synuclein and LC3-II were quantified by densitometric analysis (A, B).

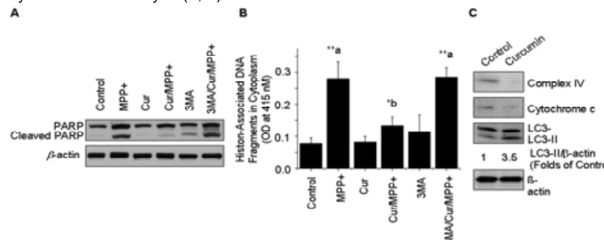

Fig. 4 Neuroprotective effect of curcumin on MPP+-induced apoptosis. Stable inducible PC12 cells expressing HA-tagged A53T mutant α-synuclein were pre-treated with curcumin for 24 h followed by addition of MPP+ (500 μM) for another 24 h. Total proteins were isolated and subjected to immunoblotting assay to determine the protein level of cleaved PARP (A), or subjected to ELISA assay to have a quantitative measurement of cytoplasmic histone-associated DNA fragments (B). Un-induced PC12 cells expressing A53T mutant α-synuclein were treated with curcumin (10 uM) for 48 h. The protein levels of mitochondrial complex IV, cytochome c were determined by immunoblotting assay with specific antibody against complex IV or cytochrome c (C). Con = Control; Cur = Curcumin.

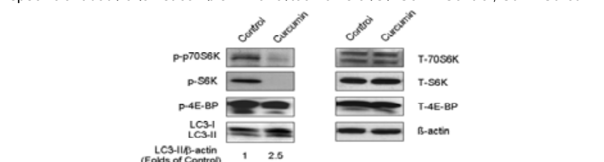

Fig. 5 Inhibition of mTOR activity by curcumin. Stable inducible PC12 cells expressing A53T mutant α-synuclein were treated with or without curcumin for 48 h followed by immunoblotting assay for evaluating mTOR activity, including levels of phosphored and total p70S6K, S6P, and 4E-BP with indicated antibodies.

Results

1. Our results showed that curcumin treatment caused an increase of LC3II in PC12 cells, indicating the induction of autophagy (Fig. 1A). The increased expression of LC3-II by curcumin was blocked when autophagy gene *Beclin 1* was suppressed by *Beclin 1* siRNA transfection (Fig. 1B). Immunoblotting assay revealed that curcumin-mediated increase of LC3-II was further enhanced with Baf1 treatment (Fig. 1C), indicating that curcumin-induced autophagy is metabolised through autophagy-lysosome pathway.

2. The protein levels of α-synuclein were increased when the stable inducible PC12 cells were treated with doxycline for 48 h as determined by immunoblotting assay (Fig. 2A). The densitometric quantification of α-synuclein bands demonstrated that the protein level of α-synuclein in doxycline-induced cells was 3.6, 3.0 and 2.0 folds of that in un-induced PC12 cells expressing wild type, A53T and A30P expression PC12 cells, respectively (Fig. 2B).

3. Curcumin treatment caused decrease of α-synuclein protein levels in A53T and A30P expressing PC12 cells accompanied by the increase of LC3-II, whereas, the reduction is not markedly shown in wild-type PC12 cells (Fig. 3A). The quantification of the density of α-synuclein bands showed that it was decreased by 8%, 72% and 63% in wild-type, A53T and A30P-expressed PC12 cells, respectively (Fig. 3B).

4. The stable inducible A53T expressing PC12 cells were differentiated with NGF (100 ng/ml) for 5 days followed by exposure to MPP+ (0.5 mM) for 24 h with or without curcumin pretreatment. The results showed that exposure of cells to MPP+ caused increase of cleaved PARP protein level as determined by immunoblotting assay (Fig. 4A) and the increase of histone-associated DNA fragments as determined by ELISA assay (Fig. 4B), which was partially blocked by curcumin pretreatment (Fig. 4A, 4B). Moreover, the neuroprotective effect of curcumin on MPP+-induced apoptosis was inhibited by autophagy inhibitor 3MA (Fig. 4A, Fig. 4B). Additionally, we found that curcumin treatment decreased mitochondrial load by showing the reduction in protein levels of mitochondrial complex IV and cytochrome c as determined by immunoblotting assay (Fig. 4C), suggesting that curcumin may enhance the degradation of mitochondria.

5. Furthermore, immunoblotting assay demonstrated that curcumin caused inhibition of mTOR activity by showing the reduction of its downstream proteins, such as p-P70-S6K, p-S6P, and p-4E-BP (Fig. 5), suggesting that mTOR signaling pathway is involved in the induction of autophagy by curcumin.

Conclusions

Our results showed that the induction of autophagy by curcumin is possibly via mTOR inhibitory pathway and that the role of curcumin in protecting against MPP+-induced apoptosis and in accelerating the clearance of mutant α-synucleins is possibly through the induction of autophagy. Our findings suggest a potential therapeutic role of curcumin in PD.

References

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