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Overexpression of F_0F_1-ATP synthase α suppresses mutant huntingtin aggregation and toxicity *in vitro*

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ABSTRACT

Huntington's disease (HD) and other polyglutamine (polyQ) neurodegenerative diseases are characterized by neuronal accumulation of the disease protein, suggesting that the cellular ability to handle abnormal proteins is compromised. As a multi-subunit protein localized in the mitochondria of eukaryotic cells, the F_0F_1-ATP synthase α belongs to the family of stress proteins HSP60. Currently, mounting evidences indicate F_0F_1-ATP synthase α may play a role in neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). Recently, ATP synthase α was reported to have protective and therapeutic roles in primary cardiacmyocytes of iron-overloaded rats by lowering ROS production. However, little is understood about the role of ATP synthase α in cell death and neurodegeneration. Here, we demonstrate that overexpression of ATP synthase α suppresses huntingtin (htt) polyQ aggregation and toxicity in transfected SH-SY5Y cell lines. Overexpression of ATP synthase α is able to protect cell death caused by polyglutamine-expanded htt. Transient overexpression of ATP synthase α suppresses the aggregate formation by estimation of polyQ aggregation, Western blot analysis, and filter trap assay (FTA) in transfected SH-SY5Y cells. These results indicated that ATP synthase α has a strong inhibitory effect on polyglutamine aggregate formation and toxicity *in vitro*, and suggest a novel neuroprotective role of ATP synthase α.

Introduction

Huntington's disease (HD) is a polyglutamine (polyQ) disease with an expanded polyQ tract in the huntingtin (htt) protein. The expansion results in the production of mutant proteins which aggregate and form insoluble inclusions within affected neurons [1]. The expansion of the CAG repeat in htt results in polyQ tract in the extreme NH_2 terminus of the protein that destabilizes the protein, leading to the formation of SDS-insoluble protein aggregates also known as inclusion bodies (IBs) *in vitro* and *in vivo* [2,3].

htt, a large protein with a molecular mass of 350 kDa lacking similarity to other proteins, has a polyQ stretch in the middle of exon 1. In normal individuals, the length of the polyQ tract is <35 repeats, but with expansion beyond 40, the htt protein is misfolded, forms aggregates, becomes toxic, and causes disease [4].

Although the mechanism by which the mutant htt (mhtt) proteins mediate neuronal cell death remains controversial, a variety of causes attribute to the toxicity of the aggregates in neurons, including impairment of axonal transport and synaptic transmission [5,6], suppression of energy metabolism [7], and induction of apoptosis [8], a defect in RNA synthesis, cell survival activity, microtubule-dependent trafficking, or the ubiquitin–proteasome system [9–15]. Previous studies have shown that inhibiting polyQ aggregation alleviates the symptoms of HD in *Drosophila* and mouse models of HD [16,17]. Therefore, it is important to delineate the aggregation mechanism of mhtt and to identify the factors that induce an environment permissive to this neurodegeneration. There is a considerable effort to find molecules that suppress polyQ aggregation and cell death/toxicity for therapeutic means [18,19].

ATP synthase, also called the F_0F_1-ATP synthase or F_0F_1-ATPase, synthesizes cellular ATP from ADP and inorganic phosphate (Pi) [20]. The F_0F_1-ATP synthase is a multi-subunit protein localized in the mitochondria of eukaryotic cells, where it utilizes the electrochemical gradient, established across the inner mitochondrial membrane by oxidative phosphorylation, for the synthesis of ATP from ADP and inorganic phosphate [21]. Like other mitochondrial enzymes, the majority of F_0F_1-ATP synthase subunits are subject to nuclear transcription, translocation from the cytosol to the mitochondria, and assembly into a macromolecular complex in the inner mitochondrial membrane.

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Previous studies have suggested the F_0F_1-ATP synthase α belongs to the family of stress proteins HSP60 [22] and thus, that it could perform similar functional role(s) to those recently described for mitochondrial HSP60 in both the mitochondria and the peroxisomes [22]. Furthermore, recent study suggested that the two conserved elements among the chaperonines and the subunits could putatively be involved in the chaperonine function of these proteins. Recent reports have also implicated the nuclear coded F_0F_1-ATP synthase α physically interacts with HSP60, which is known to stabilize numerous mitochondrial proteins [23] and HSP90.

Currently, mounting evidences indicate F_0F_1-ATP synthase α may play a role in neurodegenerative diseases, including AD and PD. For example, increased ATP synthase levels were found in the frontal cortex in PD [24]. ATP synthase has a twofold higher abundance in PD specimens [25]. ATP synthase α can bind the extracellular domain of APP and Aβ [26]. A study showed that $A\beta_{25-35}$ can induce the expression of ATP synthase α-chain in primary rat cortical neurons [27]. An interesting research showed that ATP synthase α-chain associate with neurofibrillary degeneration and accumulate in the cytosol of Alzheimer degenerating neurons in AD [28]. However, little is understood about the role of ATP synthase α in cell death and neurodegeneration.

Given that ATP synthase α have the HSP function, protective roles in primary cardiacmyocytes of iron-overloaded rats by lowering ROS production, and different HSPs play protective roles in polyQ diseases [29–37], we tested hypothesis that ATP synthase α can reduce protein aggregates and cytotoxicity of proteins containing expanded htt polyalanine tracts. We studied the role of ATP synthase α in mhtt aggregate formation in SH-SY5Y cells. The studies reported here demonstrate that ATP synthase α reduces aggregate formation and neurotoxicity in cultured neuronal cells expressing htt protein with an expanded polyglutamine tract.

Materials and methods

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Inc. Hoechst 33258 and ECL detection kit were purchased from Beyotime (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) supplement was obtained from Gibco Invitrogen Corporation. Mouse anti-ATP synthase α were from BD Biosciences. Antibody against GFP was from Millipore. All the other chemicals used were of the high grade available commercially.

Plasmid construction. Full-length ATP synthase α cDNA was PCR-amplified from mice ATP synthase α cDNA using the following oligonucleotide primers: 5′-GAGCTCAAGCTTCGAATGCTGTCCGTGCGCATCGCCGCGGCC-3′ and 5′-CGGTGGATCCCGAGGTTCAAACCCAGCCAAGAAGTTTGTT-3′. The PCR products so obtained were cloned into a $pDsRed_1$-N_1 mammalian expression vector. Sequences were confirmed by automated DNA sequencing. The functional overexpression of ATP synthase α was confirmed by Western blotting or immunohistochemistry. ATP synthase α plasmid DNA was then transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Cell culture and transient transfections. Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum at 37 °C and 5% CO_2. Cells were transfected when they reached about 70–80% confluence by Lipofectamine 2000 (Invitrogen) in accordance to the manufacturer's protocol.

Determination of cell viability. Cell viability was assessed by measuring formazan produced by the reduction of MTT. ATP syn transfected or ATP syn/polyQ co-transfected SH-SY5Y cells in 6-well culture dishes were incubated for 24 h at 37 °C. MTT was added (5 mg/ml), and the cells were incubated an additional 4 h. After this, the medium was removed and the cells were solubilized with dimethylsulfoxide and transferred to a 96-well plate. The formazan reduction product was measured by reading absorbance at 570 nm in a plate reader.

Estimation of polyQ aggregation. Cells were counted as aggregate-positive if one or several aggregates were visible within a cell as previously described [4,30].

Western blot analysis. Western blot analysis was performed as previously described [38,39].

Filter trap assay (FTA). FTA was performed as previously described [38,39].

Statistical analysis. All data were presented as means ± SEM. Data were subjected to statistical analysis via one-way ANOVA followed by Student's *t*-test with GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Mean values were considered to be statistically significant at $p < 0.05$.

Results

Transient ATP synthase α transfection induces overexpression of ATP synthase α protein in SH-SY5Y cells

ATP synthase α overexpression was achieved by transient transfections with ATP synthase α plasmid DNA (DsRed-ATP syn). SH-SY5Y cells were transfected with ATP synthase α DNA or vector DNA (DsRed) for 24 h. Following the transfection, ATP synthase α protein expression were determined by Western blotting. ATP synthase α protein expression was elevated following transfection of ATP synthase α (Fig. 1A). ATP synthase α immunoreactivity increased in the cytoplasm of SH-SY5Y cells following ATP synthase α transfection (Fig. 1B). Thus, transient ATP synthase α transfections induce overexpression of ATP synthase α protein in SH-SY5Y cells.

The ATP synthase α overexpression protects against polyQ-mediated cell death

To quantitatively examine the effects of overexpression of ATP synthase α on huntingtin toxicity, we used an MTT assay. We ob-

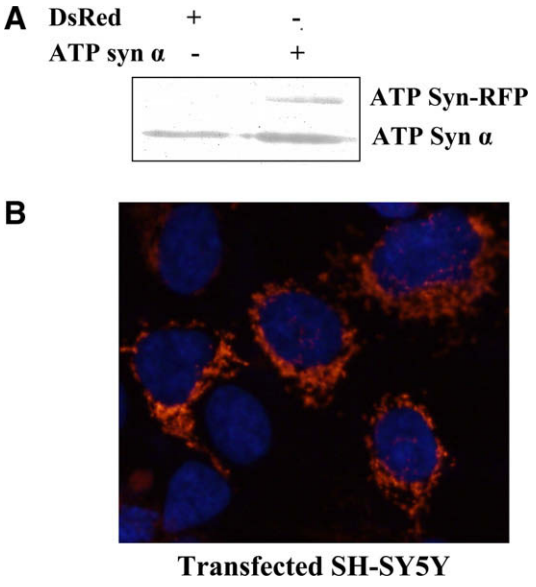

Fig. 1. ATP synthase α protein expression in SH-SY5Y cells following transfection. SH-SY5Y cells were transfected with ATP synthase α or $pDsRed_1$-N_1 DNA (DsRed) for 24 h. Following the transfection, ATP synthase α protein expression was determined by Western blotting. (A) ATP synthase α protein expression after ATP synthase α transfection. (B) ATP synthase α immunoreactivity after ATP synthase α transfection in SH-SY5Y cells.

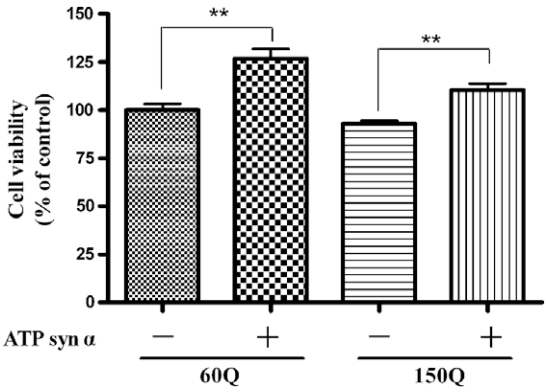

Fig. 2. Inhibition of huntingtin toxicity by the ATP synthase α protein overexpression. Cell viability was detected using MTT assay. Cotransfection of ATP synthase alpha with 60Q or 150Q for 24 h significantly increased the viability of the transfected cells, compared with overexpression of 60Q or 150Q. $^{**}p < 0.01$.

served that the transient cotransfection of 60Q or 150Q with ATP synthase α overexpression for 24 h significantly increased cell viability, compared with cotransfection of 60Q or 150Q with DsRed Vector (Fig. 2). This results indicate that ATP synthase α suppresses mhtt-induced neurotoxicity in cultured SH-SY5Y cells.

ATP synthase α suppresses huntingtin aggregation

The effect of ATP synthase α on mhtt aggregate formation was examined by transient expression in SH-SY5Y cells, frequently used for studies on mhtt aggregation. When htt with 60Q or 150Q, both displayed bright fluorescent foci (Fig. 3A). Cotransfection with ATP synthase α dramatically reduced the number of cells containing inclusions (Fig. 3B). The effect of ATP synthase α was also confirmed biochemically by immunoblot analysis. The formation of SDS-insoluble aggregates was detected in the stacking gel by immunoblotting when either 60Q or 150Q together with or without cotransfection with ATP synthase α (Fig. 4A). The SDS-insoluble aggregates reduced when 60Q or 150Q was coexpressed with ATP synthase α. Meanwhile, the decrease in insoluble form of polyQ on gel top was accompanied by an increase in the monomeric polyQ protein (Fig. 4A). We also performed filter trap assay (FTA) to further investigate the effect of ATP synthase α overexpression on Htt60Q and 150Q solubility. Cells were transfected

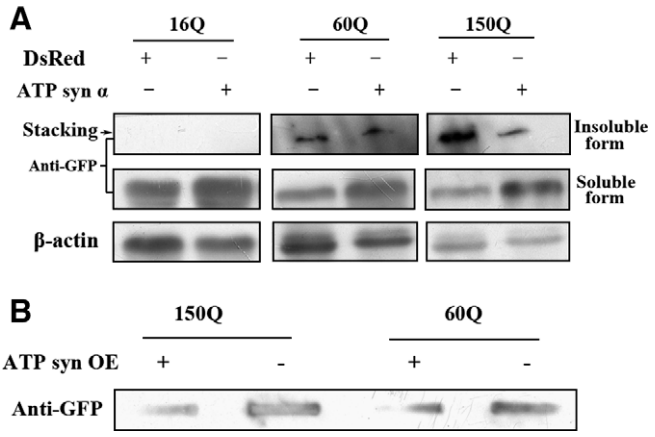

Fig. 4. Suppression of the formation of mhtt aggregation by ATP synthase α overexpression SH-SY5Y cells expressing Htt60Q and 150Q with or without ATP synthase α overexpression. (A) Western blot analysis of soluble and insoluble polyQ proteins in SH-SY5Y cells transfected with the indicated expression plasmids. Detection of SDS-insoluble aggregates by anti-GFP immunoblotting. An arrow indicates aggregates in the stacking gel. (B) Slot-blot assays were performed. The accumulation of insoluble 60Q and 150Q protein were probed with anti-GFP antibodies.

with 60Q/150Q together with or without plasmids encoding ATP synthase α. After 24 h, cells were harvested and aggregation was analyzed by slot-blot assays. As shown in Fig. 4B, the amount of aggregated protein trapped in the membrane reduced when ATP synthase α was overexpressed. This result further indicated that ATP synthase α can increase the solubility of polyQ, mirroring the suppression effect of ATP synthase α on mhtt aggregate formation in Figs. 3 and 4A. Collectively, these results indicate that ATP synthase α suppresses aggregate formation of mhtt in cultured SH-SY5Y cells.

Discussion

Huntington's disease (HD) is a polyglutamine (polyQ) disease with an expanded polyQ stretch in the NH_2 terminus of the huntingtin protein (htt). A major pathological feature of HD neurons is inclusion bodies, detergent-insoluble aggregates composed of polyQ-expanded mutant htt (mhtt). Misfolding of mhtt is thought to confer a toxic property via formation of aggregates. Although

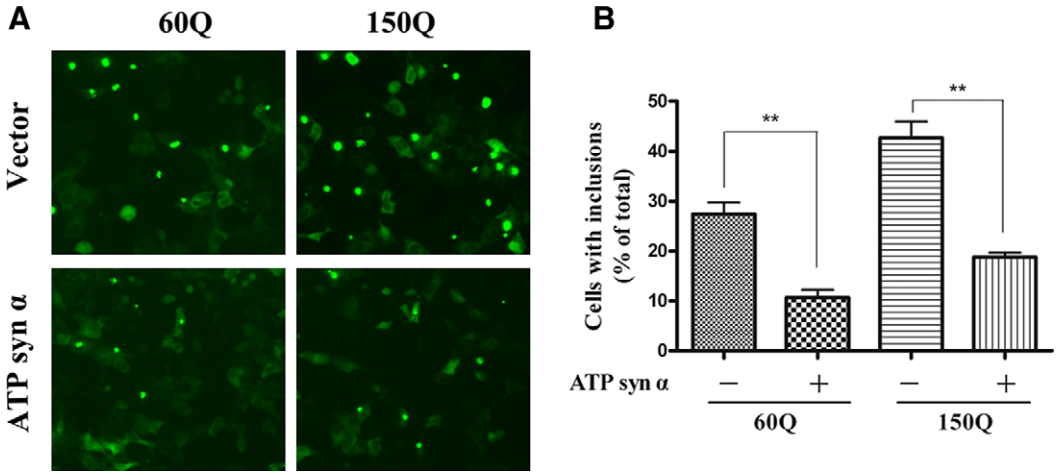

Fig. 3. Suppression of the formation of mhtt inclusions by ATP synthase α overexpression. (A) Fluorescence microscopic images of mhtt-60Q, or 150Q-EGFP expressed alone (top panels) or coexpressed with ATP synthase α (bottom panels) in SH-SY5Y cells (left panels). Cells were analyzed for inclusion formation 24 h after transfection. (B) Quantification of cells containing EGFP inclusions is shown in the right panel (mean ± SEM; $^{**}p < 0.001$).

toxic molecular species are still debated, it is important to clarify the aggregation mechanism to understand the pathogenesis of mhtt. In recent years strategies which may increase the removal of disease proteins have been examined. In this study we investigated for the first time the effect of expressing ATP synthase α on the formation of inclusions containing polyQ expansions. The results showed overexpression of ATP synthase α alone significantly reduced inclusion formation in two htt polyglutamine disease models.

We are interested in the ATP synthase α, because currently, mounting evidences indicate F_0F_1-ATP synthase α may play a role in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease. Recently, ATP synthase α was reported to have protective and therapeutic roles in primary cardiacmyocytes of iron-overloaded rats by lowering ROS production. For example, increased ATP synthase levels were found in the frontal cortex in PD [24]. ATP synthase has a twofold higher abundance in PD specimens [25]. ATP synthase alpha can bind the extracellular domain of APP and Aβ [26]. A study showed that $A\beta_{25-35}$ can induce the expression of ATP synthase α-chain in primary rat cortical neurons [27]. A interesting research showed that ATP synthase α-chain associate with neurofibrillary degeneration and accumulate in the cytosol of Alzheimer degenerating neurons in Alzheimer's disease [28]. However, little is understood about the role of ATP synthase α in cell death and neurodegeneration.

Given that ATP synthase α have the HSP function, have protective roles in primary cardiacmyocytes of iron-overloaded rats by lowering ROS production, and different HSPs play protective roles in polyQ diseases [29–37], we tested hypothesis that ATP synthase α can reduce protein aggregates and cytotoxicity of proteins containing expanded htt polyalanine tracts. We studied the role of ATP synthase α in mhtt aggregate formation in SH-SY5Y cells. The studies reported here demonstrate that ATP synthase α reduces aggregate formation and neurotoxicity in cultured neuronal cells expressing htt protein with an expanded polyglutamine tract.

Our experiments do not provide the mechanism by which ATP synthase α suppresses mutant huntingtin aggregation and toxicity *in vitro*. However, based on the properties of ATP synthase α proteins discovered so far, we speculate it may function in the following way(s). One possibility is that ATP synthase α may enhance clearance of polyalanine and polyglutamine aggregates by inhibiting oxidative stress. Because oxidative stress can promote mutant huntingtin aggregation and mutant huntingtin-dependent cell death by mimicking proteasomal malfunction [40]. Another possibility is that ATP synthase α may escort misfolded proteins, such as those containing expanded polyglutamine tracts, to the proteasome for degradation. Overexpression of ATP synthase α may accelerate the delivery of misfolded protein to the proteasome and thereby enhance their clearance. Thirdly, the character of F_0F_1-ATP synthase α as cofactors of HSP60 may assign it the function as other cofactors of HSP did, such as CHIP suppressed polyQ aggregation and toxicity in cell models of SCA1, SCA3 and Huntington's disease [39,41], a novel zebrafish model of polyQ disease [39] and a *Drosophila* model of SCA1 [42].

In summary, our data suggest that ATP synthase α suppresses the formation of insoluble htt polyQ inclusions, and reduces the toxicity-induced by htt polyQ. The mechanism by which ATP synthase α on suppression of the formation of insoluble htt polyQ inclusions, and reduction of the toxicity deserved to be investigated in the future study.

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