17-AAG Protects Against Rotenone-Induced Apoptosis in SH-SY5Y Cells *via HSP70* Induction

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Abstract

Objective To examine the protective effects of 17-AAG on rotenone-induced apoptosis in SH-SY5Y cells **Background** Heat shock proteins (HSPs), such as HSP70, represent an important cellular protective mechanism against neuronal cell death in various models of neurological disorders. 17-allylamino-17-demethoxygeldanamycin (17-AAG), an HSP70 inducer acting via Hsp90 inhibition, is now in clinical trials for a wide range of malignancies. Recent studies have suggested that 17-AAG may have a therapeutic role in non-oncological diseases as neuroprotective agent. Here, we investigated the possible role of 17-AAG in rotenone (a potent complex I inhibitor)-induced apoptosis in SH-SY5Y cells, an *in vitro* model relevant to PD. **Methods** Cells were exposed to rotenone with or without 17-AAG pretreatment. The apoptosis were determined by Hoechst 33342 staining and immunoblotting assay. The changes in mitochondrial membrane potential were determined using Mitotracker Red CMXRos staining. The release of cytochrome *c* was determined by immunoblotting assay. The protein levels of HSP70 and P53, a major protein of apoptotic signaling pathway were determined by immunobloting assay. To correlate the role of Hsp70 in the neuroprotection of 17-AAG, the induction of HSP70 was inhibited by KNK437, a novel Hsp inhibitor, or by *HSP70* siRNA transfection, followed by addition of rotenone with or without 17-AAG pretreatment. Then the apoptosis was evaluated by measuring the protein levels of cleaved PARP. **Results** 17-AAG treatment caused a time- and dose-dependent increase of HSP70 and decrease of P53. Pretreatment with 17-AAG alleviated rotenone-induced apoptosis, decreased cytochrome *c* release, and reduced the loss of mitochondria membrane potential caused by rotenone. The protective effect of 17-AAG was related to HSP70 induction and P53 inhibition, and was partially blocked by KNK437, or by *HSP70* siRNA transfection, in which the *HSP70* gene was suppressed. The attenuated accumulation of high molecular weight ubiquitin bands observed in rotenone-treated cells further support the neuroprotective effect of 17-AAG. **Conclusions** The protective effects of 17-AAG against rotenone-induced apoptosis through HSP70 induction may lead to a novel approach to neurodegenerative disorders involving mitochondrial dysfunction. Further studies are needed to examine the effect of 17-AAG in PD related animal models *in vivo* and other mechanisms involved in the neuroprotection will also be further investigated.

Background

Heat shock proteins (HSPs), such as HSP70, represent an important cellular protective mechanism against neuronal cell death in various models of neurological disorders. 17-allylamino-17-demethoxygeldanamycin (17-AAG), an HSP70 inducer acting *via* Hsp90 inhibition, is now in clinical trials for a wide range of malignancies. Recent studies have suggested that 17-AAG may have a therapeutic role in non-oncological diseases as neuroprotective agent. Human neuroblastoma SH-SY5Y cell line, subcloned from the SK-N-SH cells, is often used as a model of dopaminergic neurons. Rotenone, a widely used pesticide, has been shown to inhibit mitochondrial complex I and induces apoptosis, selectively destroys DA-ergic neurons and produces impaired motor function, characteristic of Parkinson's disease (PD) in rats. Here, we used rotenone-induced apoptosis in SH-SY5Y cells as an *in vitro* model resembling neurodegeration related to PD to demonstrate the neuroprotective effects of 17-AAG on rotenone-induced injury of cells and to explore its possible mechanisms. .

Methods

Cell culture and reagents SH-SY5Y cells were routinely grown in 10% DMEM and cultured at 37°C under humidified 5% CO2 atmosphere. Solution of rotenone was made freshly in dimethyl sulfoxide (DMSO) prior to each experiment. 17-AAG was prepared in DMSO at a stock of 1 mM and kept in -20oC.

Immunoblotting assay Total proteins isolated from the cells with mammalian tissue lysis/extraction reagent according to the manufacture's protocol were subjected to immunoblotting assay using specific antibodies as indicated. *β*-actin was used to demonstrate equal protein loading. Protein levels of cytochrome *c* were determined in both mitochondrial and cytosal fractions.

Apoptosis assay For microscopic nuclear DNA analysis, cells were stained with Hoechst 33342. The cells with condensed nuclei were considered to be apoptotic. The apoptosis was also determined by immunoblotting assay with anti-PARP antibody.

Cell live/dead assay Cell survival was quantified by using a live/dead assay kit. Cells were incubated with calcein AM and ethidium homodimer-1 (EthD-1) (1 μM) for 45 min at room temperature followed by analysis under fluorescence microscopy.

Visualization of mitochondrial membrane potential by mitotracker After specific treatment, Mitotracker Red CMXRos (500 nM) was added to the cultures for 30 min at 37°C. Then the medium containing Mitotracker was replaced with fresh PBS and the cells were fixed with 4% paraformaldehyde for 20 min. The degree of mitochondrial membrane potential was visualized quantitatively as red fluorescence, and the morphologies of nuclei stained with DAPI could be observed as blue fluorescence simultaneously.

***HSP70* siRNA Transfection** Cells were transiently transfected with small interference RNA (siRNA) of *HSP70* using lipofectamine™ 2000. Silencer™ negative control #3 siRNA was used as a sham control. The transfected cells were collected for protein isolation or were exposed to rotenone with or without 17-AAG pretreatment followed by apoptosis evaluation *via* immunoblotting assay.

Figures

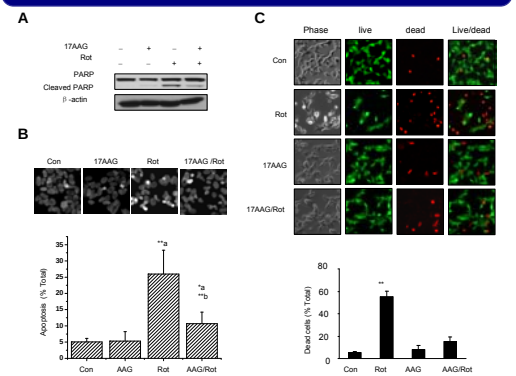

Fig.1 Neuroprotection of 17-AAG on rotenone-induced apoptosis in SH-SY5Y cells. The SH-SY5Y cells were pretreated with 17-AAG for 24h followed by addition of rotenone (10 μM) for another 24 h. Apoptosis was assessed by detection the protein levels of cleaved PARA by immunoblotting assay (A). Apoptotic cells were quantified by staining the nuclear with Hoechst 3334 (B, upper) and quantification of apoptosis were expressed as percentage of the total, and the values were the mean ± SD (B, lower). Data were analyzed by one-way analysis of variance (ANOVA). *: $p < 0.01$; **: $p < 0.05$ as compared with vehicle treated control (a), Rot group (b), (Con = control; Rot = rotenone). After specific treatment, live and dead cells were assayed by cell live/dead assay kit and determined under flurescent microscopy (C, upper). The green fluorescence, generated by calcein AM, indicates live cells, and the red fluorescence, generated by ethidium homodimer-1, indicated dead cells. The number of dead cells was counted and value was expressed as percentage of total cells. Data were expressed as the means ± SD (C, lower). **: $P < 0.01$ as compared to control.

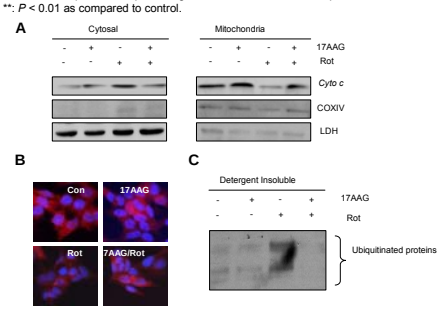

Fig.2 17-AAG attenuated rotenone-induced dysfunction of mitochondria and enhanced degradation of ubiquitinated proteins. The effect of 17-AAG on the release of cytochrome *c* was evaluated by immunoblotting assay with anti-cytochrome *c* antibody in both mitochondrial and cytosolic fractions (A). Mitotracker red CMXRos at a final concentration of 500 nM was used to visualize the mitochondrial transmembrane potential (red, B). Blue indicates DAPI nuclear staining (B). The levels of ubiquitinated proteins from detergent-insoluble fractions of cells were detected by immunoblotting assay with anti-ubiquitin antibody (C).

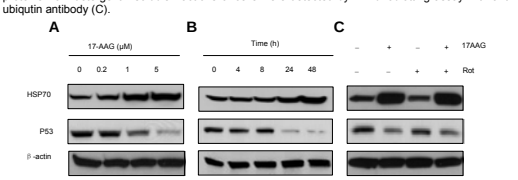

Fig.3 Expression of Hsp70 following the treatment of 17AAG. SH-SY5Y cells were treated with increasing doses of 17AAG for 48 h (A) or 5 μM 17AAG for various time durations, up to 48 h (B), or pretreated with 17-AAG followed by exposure to rotenone for another 24 h （C） before harvesting for immunoblotting assay using anti-Hsp70, anti-p53 antibodies. β-actin was used for equal loading of proteins.

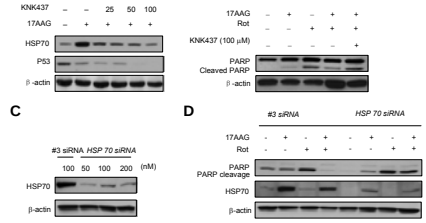

Fig. 4 Suppression of Hsp70 blocked the neuroprotection of17-AAG. Cells were treated with KNK437, an inhibitor of Hsp 70, at various concentrations 1 h before of 17-AAG treatment (A) and the effect of KNK437 on the inhibition of Hsp70 was evaluated by immunoblotting assay (A). Cells were pre-treated with 17-AAG with or without KNK437followed by adding rotenone for another 24 h and the apoptosis was assessed by detection the protein levels of cleaved PARA by immunoblotting assay (B). SH-SY5Y cells were transfected with *Hsp70* siRNA at various concentrations for 72 h. Transfection of cells with silencerTM negative control #3 siRNA was used as a sham control (C). Protein levels of Hsp70 were measured by immunoblotting assay with anti-Hsp70 antibody. Cells with negative control #3 siRNA or *Atg5* siRNA transfection were pretreated with 17-AAG for 24 h followed by addition of rotenone for another 24 h (D). The protein levels of cleaved PARP and Hsp70 were measured by immunoblotting assay. ß-actin was used as an equal loading of proteins.

Results

➢ **17-AAG prevents against rotenone-induced cell injury** Exposure of cells to rotenone caused an increase of PARP cleavage, a marker of apoptosis. However, pretreatment of cells with 17-AAG at 1 μM for 24 significantly reduced the rotenone-induced PARP fragmentations (Fig.1B). After analyzing the microscopic nuclear DNA, we found a 25% increase of apoptotic bodies in rotenone-treated cells vs. vehicle-treated control ($P < 0.01$, Fig. 1B), which was decreased to 9% with 17-AAG pretreatment (Fig. 1B). Rotenone caused shrinkage of SH-SY5Y cells, while 17-AAG pre-treatment inhibited these changes in cell morphology (Fig. 1C). Survival and death of SH-SY5Y cells were simultaneously visualized using a live/dead assay, in which, the red fluorescence indicated cell death, whereas, green fluorescence indicated cell survival. Quantification showed that the percentage of dead cells among the total was decreased from 58% with rotenone treatment to 17% with 17-AAG pretreatment (Fig. 1C).

➢ **17-AAG attenuated rotenone-induced mitochondrial dysfunction and increased clearance of aggregated proteins** The protein levels of cytochrome *c* in both mitochondrial and cytosolic fraction were detected by immunoblotting assay. Exposure of cells to rotenone caused an increase of cytochrome *c* in cytosol fraction and a decline of cytochrome *c* in mitochondria (Fig. 2A), which displacement was blocked by 17-AAG pretreatment (Fig.2A). The results that high protein levels of LDH (a specific marker for cytoplasm) were detected in cytosolic fraction and COX IV (a specific marker for mitochondria) were detected in mitochondrial fraction indicated a good purification of cytosol and mitochondria (Fig. 2A). Mitotracker Red CMXRos was used to indicate the alteration of mitochondrial membrane potential (MMP). Exposure of cells to rotenone at 10 μM for 3h caused a dramatic decrease of red fluorescence, indicating a loss of MMP (Fig. 2B), which was partially restored by 17-AAG pretreatment (Fig. 2B). Exposure of cells to rotenone caused accumulation of high molecular weight ubiquitin bands as determined by immunoblotting assay, which was partially attenuated by 17-AAG pretreatment (Fig. 2C).

➢ **Neuroprotection of 17-AAG is mediated *via* induction of HSP70**17-AAG treatment induced a significant increase of Hsp70 accompanied by the decrease of P53 in SH-SY5Y cells, which was both dose- and time-dependently as determined by immunoblotting assay (Fig. 3A, B). The increase of HSP70 and the decrease of P53 were corresponded to the decrease of cleaved PARP as shown in Fig. 1A. To correlate the role of 17-AAG to HSP70 induction, first, cells were treated with 17-AAG with or without KNK437, a novel heat shock protein inhibitor. Our results showed that the induction of HSP70 was inhibited by KNK437 dose-dependently (Fig. 4A). Then the cells were pretreated with 17-AAG and KNK437 for 24 h followed by addition of rotenone for another 24 h. The resulted proteins were subjected to immunoblotting assay and detected with anti-PARP antibody. As shown in Fig.4B, the reduction of rotenone-induced increase of cleaved PARP by 17-AAG was partially abolished by KNK437, indicating that the neuroprotection of 17-AAG was correlated to the induction of HSP70. To further investigate the potential role of HSP70 induction, we evaluated the impact of reducing HSP70 expression on the neuroprotection of 17-AAG. First, we transfected SH-SY5Y cells with *HSP70* siRNA at various concentrations or with silencerTM negative control *#3* siRNA (sham control) for 72 h. Our results showed that *HSP70* siRNA transfection efficiently and specifically reduced HSP70 protein expression in SH-SY5Y cells determined by immunoblotting assay (Fig. 4C). Then *HSP70* siRNA at 100 nM was selected for the further experiments. The SH-SY5Y cells were transfected with *HSP70* siRNA or *#3* negative control siRNA at 100 nM for two days followed by the exposure to rotenone with or without 17-AAG pretreatment. The results showed that 17-AAG attenuated the rotenone-induced increase of cleaved PARP in *#3* siRNA transfected cells, which effect was blocked in *HSP70* siRNA transfected cells (Fig. 4D), indicating that knockdown of HSP70 expression in SH-SY5Y cells blocked the role of 17-AAG in preventing rotenone-induced apoptosis.

Conclusions

➢The protective effects of 17-AAG against rotenone-induced apoptosis through HSP70 induction may lead to a novel approach to neurodegenerative disorders involving mitochondrial dysfunction.

➢Further studies are needed to examine the effect of 17-AAG in PD related animal models *in vivo* and other mechanisms involved in the neuroprotection will also be further investigated.

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