Resveratrol, a Polyphenol Found in Red Wine, Protects Against Rotenone-induced Apoptosis Through Autophagy Induction

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Abstract

Objective: To examine the neuroprotective effects of resveratrol on rotenone-induced apoptosis in SH-SY5Y cells. **Background:** Resveratrol, an antioxidant polyphenol found in red wine, has been reported to increase the expression of mammalian Sir2 deacetylase (SIRT1). The fact that SIRT1 can inhibit stress-induced apoptotic cell death and the report that SIRT1 is an important regulator of autophagy led us to hypothesize that resveratrol may enhance autophagy through SIRT1 and prevent neuronal death related to accumulation of aggregated/misfolded proteins found in several age-related neurodegenerations, including PD. Rotenone is an inhibitor of mitochondrial complex I and induces neuronal death accompanied by the inhibition of proteasome activity, which, in turn, leads to an increase in aggregated/misfolded proteins associated with the pathogenesis of PD. Here, the neuroprotective effects of resveratrol on rotenone-induced injury were examined in SH-SY5Y cells. **Methods:** The SH-SY5Y cells were treated with resveratrol at various concentrations for different time periods, or exposed to rotenone with or without resveratrol pretreatment. Protein levels of SIRT1, LC3, a marker of autophagy, or PARP, a marker of apoptosis, were determined by immunoblotting assay. The induction of autophagy was verified by electron microscopy. The cells were then transfected with *SIRT1* siRNA or *Atg5* siRNA followed by addition of rotenone with or without resveratrol. Apoptosis was evaluated by measuring the protein levels of cleaved PARP. **Results:** SIRT1 and LC3-II/I ratio were increased by resveratrol treatment indicating induction of autophagy. Electron microscopy analysis confirmed active autophagy in cells treated with resveratrol. Suppression of *SIRT1* gene blocked induction of autophagy by resveratrol. Reveratrol pretreatment attenuated the apoptosis and accumulation of aggregated proteins caused by rotenone. The neuroprotective effect of resveratrol on rotenone-induced apoptosis was partially blocked when *SIRT1* or *Atg5* gene was suppressed. **Conclusions:** In addition to the induction of SIRT1, autophagy induction by resveratrol plays an important role in protecting against rotenone-induced apoptosis, which may lead to a novel therapeutic approach to neurodegenerative disorders associated with aggregation of misfolded proteins. Further studies, including *in vivo* experiments, are needed to confirm the role of resveratrol as a putative neuroprotective agent.

Background

Resveratrol, an antioxidant polyphenol found in red wine, has been reported to increase the expression of mammalian Sir2 deacetylase (SIRT1). The fact that SIRT1 can inhibit stress-induced apoptotic cell death and the report that SIRT1 is an important regulator of autophagy led us to hypothesize that resveratrol may enhance autophagy through SIRT1 and prevent neuronal death related to accumulation of aggregated/misfolded proteins found in several age-related neurodegenerations, including PD. Rotenone is an inhibitor of mitochondrial complex I and induces neuronal death accompanied by the inhibition of proteasome activity, which, in turn, leads to an increase in aggregated/misfolded proteins associated with the pathogenesis of PD. Here, the neuroprotective effects of resveratrol on rotenone-induced injury were examined in SH-SY5Y cells.

Methods

Cell culture and treatments 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. Resveratrol was prepared in DMSO at a stock of 50 mM. To study the putative neuroprotective effects of reveratrol, cells were pretreated with resveratrol for 24 h followed by the addition of rotenone for another 16 h.

Immunoblot assay After specific treatment, total proteins were isolated with mammalian tissue lysis/extraction reagent and the equal amounts of protein were separated on SDS−polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. After being blocked in 6% nonfat dry milk for 45 min, membranes were then incubated with specific primary antibody. Chemilluminescence enhancement was performed using the corresponding secondary antibody and signals were detected using ECL. The protein brands were quantified by densitometric analysis using a GS-700 Densitometer when necessary.

Autophagy detection The induction of autophagy by resveratrol was evaluated by detecting an increased ratio of LC3 II/I by immunoblotting assay, and was also confirmed by transmission Electron Microscopy analysis.

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

Apoptosis assay Apoptosis was determined by quantitatively measurement of cytoplasmic histone-associated DNA fragments using ELISA kit, and by detection the protein levels of cleaved PARP fragments with anti-PARP antibody that recognizes both uncleaved and cleaved PARP by immunoblot assay.

Transfection of cells with GFP-LC3 plasmid or *Atg5* or *SIRT1* siRNA The cells were transiently transfected with GFP-LC3 plasmid or small interference RNA (siRNA) of *Atg5* or *SIRT1*, using lipofectamineTM 2000 followed by resveratrol treatment with or without rotenone. The GFP-LC3 transfected cells were visualized under immunofluorescent microscopy. The siRNA transfected cells were harvested and the resulted proteins were subjected to immunoblotting assay.

Figures

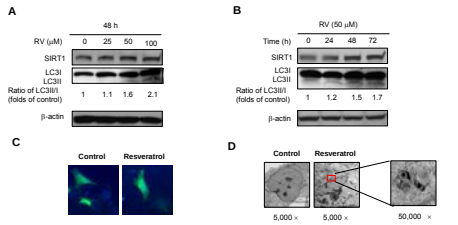

Fig.1 Resveratrol enhanced autophagy in SH-SY5Y cells. The cells were treated with resveratrol at various concentrations for 48 h (A) or at 50 μM for different time durations (B). The protein levels of SIRT1, LC3 were determined by immunoblotting assay with anti-SIRT1 or anti-LC3 antibody. Cells transfected with GFP-LC3 expression plasmid were treated with resveratrol for 48 h followed by analysis under fluorescent microscopy (C). After treatment with resveratrol for 48 h, the cells were fixed and the double membrane structrure of autophagy vacuous were analysed by electron microscope under a JEM 1010 transmission electron microscope (D).

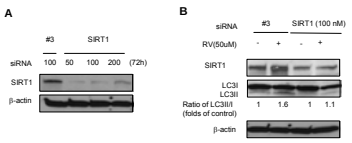

Fig.2 SIRT1-dependent induction of autophagy by resveratrol. Transfection of cells with SIRT1 siRNA significantly inhibited the protein levels of SIRT1(A). The cells with or without SIRT1 siRNA transfection were treated with resveratrol for 48 h. The result proteins were subjected to immunoblotting assay (B)

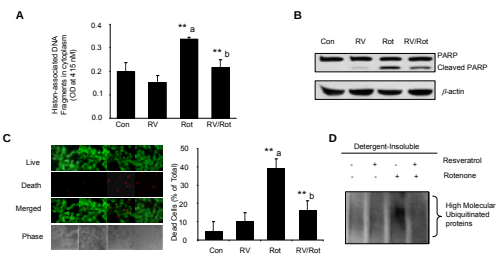

Fig.3 Resveratrol protected against rotenone-induced apoptosis and enhanced degradation of aggregated proteins in SH-SY5Y cells. Cells were exposed to rotenone with or without resveratrol pretreatment. Total proteins isolated from the cell were subjected to ELISA assay to determine the levels of histone-associated DNA fragmentation (A), or were subjected to immunoblotting assay to determine the protein levels of cleaved PARP with PARP antibody (B). ß-actin was used as an equal loading of proteins. The morphology changes and accordingly the changes of live and dead cells were detected using cell live/dead assay kit (C). 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 (C). Data were expressed as the means ± SD. **: $P < 0.01$ as compared to control (a) or rotenone (b). The high molecular bands of ubiquitinated proteins in detergent-insoluble fractions were detected by immunoblotting assay with anti-ubiquitin antibody (D).

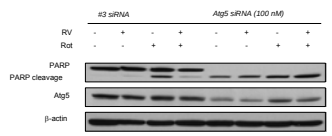

Fig.4 Neuroprotection of resveratrol on rotenone-induced apoptosis through autophagy induction. The cells transfectd with Atg5 siRNA or negative control #3 siRNA were pretreated with resveratrol followed by addition of rotenone for another 16 h. The total proteins isolated from the cells were subjected to immunoblotting assay with anti-PARP or anti-Atg5 antibody. ß-actin was used as an equal loading of proteins.

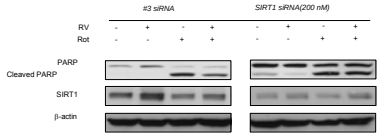

Fig.5 Neuroprotection of resveratrol on rotenone-induced apoptosis partially through SIRT1. The cells transfectd withSIRT1 siRNA or negative control #3 siRNA were pretreated with resveratrol followed by addition of rotenone for another 16 h. The total proteins isolated from the cells were subjected to immunoblotting assay with anti-PARP or anti-SIRT1 antibody. ß-actin was used as an equal loading of proteins.

Results

➢**Resveratrol enhanced autophagy in SH-SY5Y cells** Immunoblotting assay revealed that resveratrol treatment caused an increase of LC3-II /I ratio dose- and time-dependently as compared with vehicle control-treated cells (Fig. 1A, B), indicating that resveratrol enhanced autophagy in SH-SY5Y cells. The cells transfected with GFP-LC3 expression vector were treated with resveratrol for 48 h followed by fluorescent microscopy analysis. The results showed that GFP-LC3 transfected cells presented a diffuse distribution under control conditions, whereas a punctate pattern of GFP-LC3 expression (GFP-LC3 dots) was induced by resveratrol treatment (Fig. 1C), also indicating an enhancement of autophagy by resveratrol. Quantification of LC3B-punctuated cells (defined as "autophagic cell") revealed that compared with vehicle control, treatment with resveratrol was associated with a significant increase in the percentage of autophagic cells in SH-SY5Y cells (15% versus 2%, $P < 0.01$; Fig. 1C). The autophagosome and autophagolysosome, collectively referred to as autophagic vacuoles (AVs), are considered as the characteristic components of autophagy. To verify that the observed changes in LC3 reflected increased double membrane structures of AVs, the cells were studied by transmission electron microscopy. As shown in Fig. 1D, the structures of AVs could be observed largely in resveratrol-treated cells. To correlate the induction of autophagy to SIRT1 expression, the cells were transfected with *SIRT1* siRNA followed by addition of resveratrol. SIRT1 siRNA transfection caused a significant decrease of SIRT1 expression (Fig. 2A), and at same time, reveratrol-induced enhancement of autophagy was partially inhibited due to the SIRT1 suppression (Fig. 2B).

➢ **Resveratrol prevents against rotenone-induced cell injury** Exposure of cells to rotenone resulted in an increase of histone-associated DNA fragmentation (Fig. 3A) and an increase of cleaved PARP protein level (Fig. 3B), both of which are characteristics of apoptosis and were attenuated by resveratrol pretreatment (Fig. 3A, B). Rotenone caused shrinkage of SH-SY5Y cells, while resveratrol pre-treatment inhibited these changes in cell morphology (Fig. 3C). Rotenone treatment caused an increase of red fluorescent signal (indicating dead cells) and a decrease of green fluorescent signal (indicating live cells) (Fig. 3C). Resveratrol pretreatment salvaged cells from rotenone-toxicity as demonstrated by the reduction of red cells caused by rotenone treatment ($P < 0.01$; Fig. 3C). The ubiquitinated proteins in detergent-insoluble fraction of rotenone-treated cells with or without resveratrol pretreatment were detected by immunoblot assay. The accumulation of high molecular weight ubiquitin bands was observed in detergent-insoluble fraction of cells treated with rotenone, which was partially attenuated by resveratrol pretreatment (Fig. 3D).

➢**Neuroprotection of resveratrol partially mediated by the induction of autophagy and increased expression of SIRT1** To investigate the potential role of autophagy enhanced by resveratrol, we evaluated the impact of reducing autophagy gene Atg5 expression on the neuroprotection of resveratrol. The result showed that suppression of autophagy gene Atg5 increased susceptibility of cells to rotenone-induced apoptosis and partially blocked the protective effect of resveratrol on rotnone-induced apoptosis (Fig.4). To evaluate the role of resveratrol-induced increase of SIRT1, the SIRT1 gene was suppressed by transfection of cells with SIRT1 siRNA followed by exposure to rotenone with or without resveratrol pretreatment. The result showed that suppression of SIRT1 partially blocked the role of resveratrol in rotnone-induced apoptosis (Fig.5).

Conclusions

➢Here, for the first time, we reported that resveratrol may enhance autophagy in SH-SY5Y cells, which may mediated *via* SIRT1.

➢We found that resveratrol protected against rotenone-induced apoptosis and enhanced clearance of ubiquitinated proteins partially through autophagy induction.

➢We hypothesis that as a nature-derived compound, resveratrol may favorably modify the progression of PD and other neurodegenerative diseases associated with aggregation of misfolded proteins. Therefore, further studies, including *in vivo* experiments, are needed to confirm the role of resveratrol and its possible mechanisms as a putative neuroprotective agent.

Acknowledgement

This study was supported by:
Diana Helis Henry Medical Research Foundation (2007-2009);
Carolyn Weiss Law seed funding (2008-2010).