

## ABSTRACT

The progressive loss of dopaminergic neurons in substantia nigra (SN) accompanied by the formation of inclusion bodies is the pathologic hallmark of Parkinson's disease (PD). Dysfunction of ubiquitin-proteasome system (UPS) to degrade misfolded/aggregated proteins has been implicated in the pathogenesis of PD. Besides UPS, autophagy-lysosome pathway (ALP) is another important pathway to degrade misfolded/aggregated proteins. It has been shown that autophagy deficiency may lead to the development of neurodegenerative diseases. We hypothesize that induction of autophagy may have beneficial effects to support cell survival during UPS dysfunction. Deferoxamine (DFO) is one of iron chelators and has been shown to exert neuroprotective effect on lactacystin (a proteasome inhibitor)-induced injury. Since DFO has been used experimentally to mimic the effects of hypoxia which may induce autophagy, in this study, we determined the role of DFO in the induction of autophagy and verified the protective effect of DFO on rotenone (a mitochondrial complex I inhibitor)-induced apoptosis on SH-SY5Y cells, an *in vitro* model of PD. Our results showed that DFO treatment enhanced autophagy, increased the protein levels of HIF-1 $\alpha$  and GLUT1. The rotenone-induced apoptosis and the increase of the ubiquitinated protein levels were reduced with DFO pretreatment. Knock out of autophagy related genes, such as *Becn1*, enhanced rotenone-induced apoptosis and the neuroprotective effect of DFO was partially inhibited. We concluded that DFO is a potentially useful neuroprotective agent and, in addition to the induction of HIF-1 $\alpha$ , autophagy enhancement may be one of the possible mechanisms involved in the neuroprotection.

## BACKGROUND

Parkinson's disease (PD) is one of the most neurodegenerative disorders, characterized with loss of dopaminergic neurons accompanied by aggregated/misfolded protein. There are two most important protein degradation systems: ubiquitin proteasome system (UPS), and autophagy-lysosome pathway (ALP). It has been reported that once UPS is inhibited, autophagy is up-regulated and the remaining aggregated proteins are targeted by autophagy, indicating that autophagy is a default pathway for UPS. But with the pathogenic condition deterioration, this compensatory auto-regulatory mechanism finally is unable to clear the excessive misfolded proteins and then is unable to maintain the cellular balance and eventually results in neuronal death [Pan et al. 2008]. Furthermore, it has been reported that autophagy is an important process in a variety of human diseases caused by toxic, aggregate-prone, intracellular proteins, which become inaccessible to the proteasome when they form oligomers [Ravikumar et al. 2002; 2004; Webb et al. 2003; Rubinstein et al. 2007]. Thus, we hypothesized that induction of autophagy may be beneficial for the survival of cells under the condition of UPS dysfunction. Deferoxamine (DFO) is an iron-chelating agent, and has been shown to exert neuroprotective effects on lactacystin (an inhibitor of proteasome)-induced injury related to PD [Zhang et al. 2005]. Meanwhile, DFO has also been used experimentally to mimic the effects of hypoxia [Luwor et al. 2005], which may induce autophagy [Lum et al. 2005]. Therefore, in this study, first, we determined role of DFO in the induction of autophagy in SH-SY5Y cells (human dopaminergic neuroblastoma cells). Then, we verified the protective effect of DFO on rotenone (a mitochondrial complex I inhibitor)-induced apoptosis in SH-SY5Y cells, an *in vitro* model of PD [Pan et al. 2005]. We concluded that autophagy enhancement may be one of the mechanisms involved in the neuroprotection of DFO.

## METHODS

**Cell culture and transfection** SH-SY5Y cells were routinely grown in DMEM supplemented with 10% FBS and cultured at 37°C under humidified 5% CO<sub>2</sub> atmosphere. To observe the neuroprotective effect of DFO against rotenone-induced injury, SH-SY5Y cells were pretreated with DFO for 3 h followed by addition of rotenone (10  $\mu$ M) for another 24 h. To elucidate the possible mechanisms involved in the neuroprotection of DFO, the cells were treated with DFO at various concentrations for specific time durations. DFO and rotenone were dissolved in water and added to the culture medium to achieve their final concentrations. All chemicals were obtained from Sigma. To determine if DFO has similar effect to hypoxia in SH-SY5Y cells, the cells were cultured in hypoxia (1% O<sub>2</sub>) conditions or transfected with HIF-1 $\alpha$ /ODD mutant pDNA3 expression construct (a kind gift from Dr. Fan, University of Texas, MD Anderson Cancer Center, Houston, TX) using Lipofectamine 2000. The induction of HIF-1 $\alpha$  will be measured among them by immunoblotting assay. The cells were transiently transfected with small interference RNA (siRNA) of *Becn1* (RdSeq Number: NM\_00103417; NM\_053739; siRNA ID: 195717; Ambio INC, Austin, TX, USA), a principal regulator in formation of autophagosome and initiation of autophagy through class III PI3K pathway. The cells transfected with #3 siRNA were as negative control.

## RESULTS

**Proteasome activity assay** After the cells were treated with rotenone for different time duration, the cells were harvested and total proteins were isolated with lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 2 mM ATP, and 1% Triton X-100). The lysates were centrifuged at 10,000 g at 4°C for 20 min. The protein concentrations were assayed from the resulting supernatants by the Bradford's method (Bio-Rad, Hercules, CA). The proteasome activity was detected using 20S proteasome activity assay kit according to the manufacture's instruction. The results were shown as percentage of control.

**Immunoblotting assay** After specific treatment, total proteins were isolated from the cells or tissues with mammalian tissue lysis/extraction reagent (Sigma-aldrich, St. Louis, MO, USA) according to the manufacture's protocol. Equal amounts of protein were subjected to immunoblotting assay using specific antibodies: anti-poly (ADP-ribose) polymerase (PARP) antibody, anti-HIF-1 $\alpha$  antibody, and anti-GLUT1 antibody. The induction of autophagy was assessed by detecting an increase of the autophagosomal membrane form of microtubule-associated protein 1 light chain 3 (LC3), a specific marker of autophagy with anti-LC3B antibody. There are two cellular forms of the LC3 protein. One is LC3-I (18 KD), a cytoplasmic form of LC3, and another one is LC3-II (16 KD), a cleavage form of LC3, which is associated with the autophagosomal membrane. Thus, the increased ratio between LC3-II and LC3-I is associated with autophagy induction. The ratio of LC3B-II/I was evaluated by densitometric analysis and data were expressed as folds of control. To prepare the whole lysates, the cells were harvested and resuspended in 2  $\times$  sodium dodecyl sulfate (SDS) sample buffer (Biorad) and boiled for 10 min till the samples becoming clear again. Equal volume of sample lysate was conducted to immunoblot assay with a rabbit anti-ubiquitin antibody (1:2000; BIOMOL international, LP, PA, USA).

### Rotenone-induced apoptosis in SH-SY5Y cells

After the cells were treated with rotenone, the protein levels of cleaved PARP were increased time-dependently (Fig. 1A), indicating the occurrence of apoptosis in SH-SY5Y cells. The ratio of LC3BII/I was increased in cells treated with rotenone for 3 h and was back to the base level thereafter (Fig. 1A), supporting the previous report that enhanced oxidative stress possibly activates autophagy during the early stage of mitochondrial dysfunction and helps to resist the enhanced oxidative stress [Gonzalez-polo et al. 2007]. The proteasome activities were significantly decreased by 14% ( $p < 0.05$ ), 19%, 32%, 31%, 32%, 38% and by 24%, 31%, 39%, 32%, 38% and by 24%, 31%, 39%, 32%, 38% and by 24%, 31%, 39%, 32%, 38% in cells treated with rotenone at 5  $\mu$ M or 10  $\mu$ M for 3, 8, 16 and 24 h (Fig. 1B), which was more significant in cells treated with rotenone at higher concentration. Meanwhile, an accumulation of high molecular weight ubiquitin band was observed in cells treated with rotenone for 24 h (Fig. 1C).

### Induction of HIF-1 $\alpha$ , GLUT1 and autophagy by DFO in SH-SY5Y cells

HIF-1 $\alpha$ /ODD is a mutant HIF-1 $\alpha$  construct in which the ODD domain of HIF-1 $\alpha$  is deleted, and thereby, its transcriptional activity is largely retained. The increase of HIF-1 $\alpha$  level in cells treated with DFO in normoxic conditions is similar to the cells cultured in the hypoxia chamber (Fig. 2A), or in the cells transfected with HIF-1 $\alpha$ /ODD (Fig. 2A), supporting the reports that DFO may mimic the effects of hypoxia. DFO treatment increased the expression of HIF-1 $\alpha$  and GLUT1, which was both time- and dose-dependent as determined by immunoblotting assay (Fig. 2B, C). The ratio of LC3B-II/LC3B-I began to increase 16 h after DFO treatment when the dose of DFO at 100  $\mu$ M (Fig. 2B, C).

### Protective effect of DFO on rotenone-induced apoptosis

Rotenone treatment was associated with shrinking of the cell bodies which was partially blocked by pretreatment with DFO (Fig. 3A). The increased protein level of PARP cleavage fragments induced by rotenone was partially inhibited by DFO treatment as determined by immunoblotting assay (Fig. 3B). The reduced protein level of cleaved PARP was associated with the increased protein levels of HIF-1 $\alpha$ , GLUT1 and increased ratio of LC3B-II/LC3B-I after DFO treatment (Fig. 3B). To test the effect of DFO on rotenone-induced proteins aggregation in SH-SY5Y cells, ubiquitinated proteins in the whole lysate of cells were determined using immunoblot assay. The accumulation of high molecular weight ubiquitin bands was observed in rotenone-treated cells, which was partially attenuated by DFO pretreatment (Fig. 3C), indicating that DFO increased clearance of aggregated proteins.

### Genetic suppression of autophagy enhanced susceptibility of cells to rotenone

To evaluate the role of autophagy in the condition of rotenone treatment, the autophagy gene *Becn1* was genetically suppressed by transfecting the cells with *Becn1* siRNA. The protein level of *Becn1* was suppressed when the cells were transfected with *Becn1* siRNA at 100 nM for 72 h (Fig. 4A). The cells transfected with *Becn1* or #3 siRNA (as a negative control) were pre-treated with DFO for 3h followed by rotenone (10  $\mu$ M) for additional 24 h. Results showed that rotenone-induced apoptosis was more significant in cells when autophagy related gene *Becn1* was inhibited by *Becn1* siRNA transfection (Fig. 4B). The protective effect of DFO on rotenone-induced apoptosis was inhibited in cells transfected with *Becn1* siRNA by showing the continuous increase of the protein levels of PARP cleavage in *Becn1* siRNA transfected cells treated with DFO and rotenone (Fig. 4B).

## FIGURES

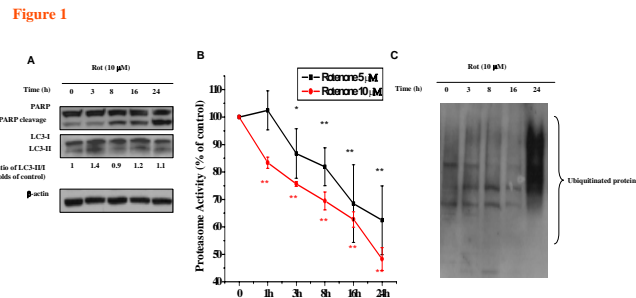


Figure 1

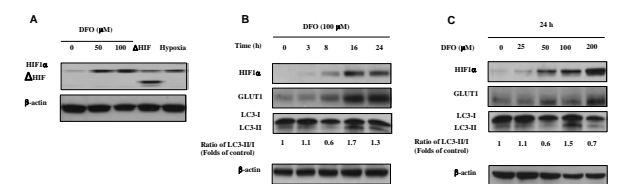


Figure 2

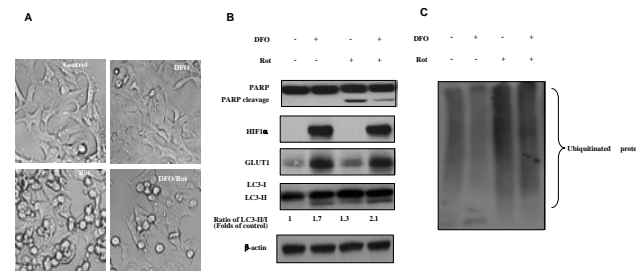


Figure 3

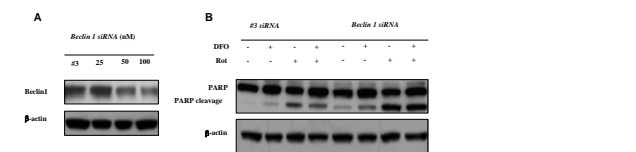


Figure 4

## LEGENDS

**Figure 1** Rotenone-induced injury in SH-SY5Y cells. The SH-SY5Y cells were treated with rotenone (10  $\mu$ M) for indicated time duration. The apoptosis of cells was determined by immunoblotting assay with anti-PARP antibody (A) and the induction of autophagy was detected with anti-LC3 antibody (A).  $\beta$ -actin was used as an equal loading of protein (A). The proteasome activities were measured in cells treated with rotenone at various concentrations for different time durations (B). The results were expressed as percentage of control. Data were the mean  $\pm$  SD values. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  as compared with control. After being treated with rotenone for different time duration, the levels of ubiquitinated proteins in whole lysate of cells were detected by immunoblotting assay with anti-ubiquitin antibody (C).

**Figure 2** Protein induction by DFO treatment. The SH-SY5Y cells were treated with DFO, or transfected with mutant HIF1 $\alpha$  pDNA3 expression construct, or in the condition of hypoxia, the protein levels of HIF1 $\alpha$  were measured by immunoblotting assay (A). The protein levels of HIF1 $\alpha$ , GLUT1, LC3 in cells treated with DFO at various concentrations for 24 h (B) or treated with DFO at 100  $\mu$ M for different time durations (C) were measured by immunoblotting assay.

**Figure 3** Effect of DFO on rotenone-induced apoptosis in SH-SY5Y cells. The cells were pretreated with DFO for 3 h followed by addition of rotenone (10  $\mu$ M) for another 24 h. The cell morphology were shown in A. The protein levels of PARP cleaved fragments were determined by immunoblotting assay with anti-PARP antibody (B) and the protein levels of HIF1 $\alpha$ , GLUT1, LC3 were measured with its specific antibody (B). The levels of ubiquitinated proteins in whole cell lysate were detected by immunoblotting assay with anti-ubiquitin antibody (C).

**Figure 4** Genetic inhibition of autophagy increased vulnerability of cells to rotenone-induced injury. The cells were transfected with *Becn1* siRNA at various concentrations for 72h. Inhibition of *Becn1* protein expression by siRNA transfection was measured by immunoblotting assay with anti-*Becn1* antibody (A). Cells with or without *Becn1* siRNA transfection were pre-treated with DFO for 3 h followed by addition of rotenone for another 24 h. The apoptosis of cells was determined by immunoblotting assay with anti-PARP antibody (B).

## CONCLUSIONS

\* DFO, one of iron chelators, provides neuroprotection against rotenone-induced apoptosis in SH-SY5Y cells and the mechanisms involved in the neuroprotection may be partially contributed to the enhancement of autophagy through enhanced degradation of misfolded proteins. Induction of HIF-1 $\alpha$  or GLUT1 may be another important neuroprotective mechanism of DFO.

\* Suppression of autophagy related gene *Becn1* enhances cellular susceptibility to rotenone-induced apoptosis, further supporting the notion that autophagy is particularly crucial for protecting cells from various insults.

\* Although there is an induction of autophagy in the early stage of insults, such as rotenone treatment, it is only a temporary compensatory auto-regulation. With the insults continuously, this auto-regulation can not afford the excessive aggregated proteins and will not be able to maintain the cellular balance and eventually results in cell death. We believe that additional enhancement of autophagy may be necessary in supporting the survival of cells under various insults, especially under the situation relevant to aggregated and misfolded proteins.

## REFERENCES

Lum JJ, et al. Nature Reviews Molecular Cell Biology 2005; 6: 439-48.  
 Luwor RB, et al. Oncogene. 2005;24:4433-41.  
 Pan T, et al. Brain 2008; Jan. 10  
 Pan T, et al. FEBS Lett 2005;579:6716-20.  
 Zhang X, et al. Biochem Biophys Res Commun 2005;333:544-9.  
 Ravikumar B, et al. Hum Mol Genet 2002; 11: 1107-17.  
 Ravikumar B, et al. Nat Genet 2004; 36: 585-95.  
 Rubinstein DC, et al. Nat Rev Drug Discov 2007; 6: 304-12.  
 Gonzalez-polo RA, et al. Autophagy. 2007; 3: 366-7.