Mechanistic study of proteasome inhibition-induced iron misregulation in dopamine neuron degeneration

Xuping Li, Wenjie Xie, Joseph Jankovic, Weidong Le

Department of Neurology, Baylor College of Medicine, Houston, TX, USA

ABSTRACT

Ubiquitin proteasome system (UPS) impairment and iron misregulation have been implicated in dopamine (DA) neuron degeneration in Parkinson's disease (PD). To investigate their involvement in DA neuron degeneration, we generated an in vitro model by applying proteasome inhibitor lactacystin in DAergic cell line MES23.5 culture. We found that lactacystin caused a marked increase in labile iron, reactive oxygen species and ubiquitin-conjugated protein aggregation prior to cell injury. IRP2 disruption resulted in an increase in transferrin receptor 1(TfR1), and a decrease in ferritin heavy chain (H-Frt), and eventually cell death. This study unravelled a mechanistic interplay between UPS impairment and iron misregulation resulted from disturbance in IRP2, TfR1 and H-Frt. Our findings provide new insight into the pathogenesis of PD and potential iron-targeted neuroprotective strategy.

BACKGROUND

We previously reported an elevated iron level, accompanied by increase in the protein levels of iron regulatory protein 2 (IRP2) and divalent metal transporter 1 (DMT1) in the SN of UPS-impaired mice. Our additional study showed that iron chelation not only alleviated neurodegeneration but also restored proteasome activity. Thus, a detailed investigation of the UPS impairment-mediated molecular pathways in PD cell model is of importance not only for better understanding of iron misregulation in PD but also for defining the complex molecular interplay between UPS and iron and its role in the pathogenesis of PD. In this study, we explore iron misregulation in the UPS impairment-induced neurodegeneration in proteasome inhibitor lactacystin treated DA cell line MES23.5 by examining the profiles and dynamic changes in iron metabolism/regulation-related genes and proteins, assessing liable iron level, ROS, protein aggregation and proteasome activity, through which we identified the key proteins involved in neuronal injury.

METHODS

Cell cultures and treatment

MES23.5 is a DA cell line developed in our laboratory. The cells were cultured in DF12 with 2% SATO supplement. Some cells were treated with the irreversible proteasome inhibitor lactacystin (5μM) or co-treated with iron chelators desferoxamine, bipyridil, antioxidant N-acetyl cysteine, DMT1 inhibitor Ebselen at the indicated concentrations, or anti-transferrin receptor 1 antibody in accordance with our experimental schedule.

Cell viability assay

MES23.5 cells were seeded on 96 well-plate, grown for 24 hr, and then pretreated respectively with vehicle, DFO, NAC, Ebs or anti-TfR1 antibody followed by exposure to lactacystin (5mM) for 12 h or 24 h. MTT (0.5mg/ml) was used to detect the viability.

Detection of labile iron and ROS

Calcein fluorescence signal can be quenched by liable iron, therefore intracellular liable iron was measured by calcein fluorescence quenching according to previously described methods. ROS was detected by using CM-H2DCFDA according to the manufacture's instructions with a few modification.

Proteasome activity assay

The 20S Proteasome Activity kit was carried out with 50 μg of cell lysates and the appropriate substrate for incubation. The activity was measured by detection of the fluorophore AMC.

Western blot assay

Proteins of MES23.5 cells were harvested according to the previous description. SDS-PAGE and transblot were followed by probed primary antibodies included: anti-IRP1, anti-IRP-2, anti-TfR1, anti-DMT1, anti-Fp1, anti-ubiquitin. Peroxidises-conjugated second antibodies, and ABS detection system were used.

RT-PCR

Iron regulation related genes, including H-Frt, L-Frt, TfR1, DMT1, Fp1, Hp, HO-1 and VEGF1□a were amplified using RT-PCR. 18s rRNA was set as internal standard.

Lentivirus ShRNA gene knockdown

To investigate the role of DMT1 and IRP2 in DA cell injury, lentvirus-based shRNA interference was used to knock down DMT1 and IRP2 in MES23.5 cells.

RESULTS

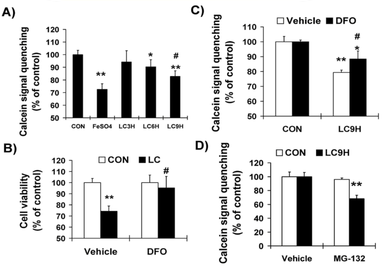

Fig. 1. Labile iron is increased in MES23.5 cells following proteasome inhibition. (**A**) Detection of labile iron pool using calcein signal quenching method. CON, vehicle treatment; LC3H/6H/9H, lactacystin treatment for 3/6/9 hr. *P <0.05, **P <0.01 vs CON; #P <0.05 vs LC3H. (B) DFO protects against lactacystin-induced cell injury (5 mM, 24 hr). **P <0.01 vs CON; #P <0.05 vs LC/Vehicle. (C) DFO attenuates the labile iron increase. *P <0.05, **P <0.01 vs CON; #P <0.05 vs vehicle/LC. (D) Increased labile iron can be reproduced by another proteasome inhibitor MG-132 (2 mM). **P < 0.01 vs CON.

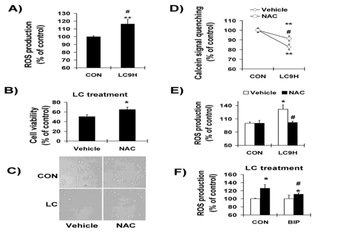

Fig. 2. Increased labile iron and consequentially generated ROS are involved in the cell injury. (A) ROS production in MES23.5 cell is increased at early stage. **P <0.01 vs CON. (B+C) The antioxidant NAC (10 mM) protects cell injury of MES23.5 cells. *P <0.05 vs CON. (D+E) NAC attenuates the increased labile iron level and the increased ROS in MES23.5 cells. **P <0.01 vs CON; #P <0.05 vs vehicle/LC. (F) Iron chelation with BIP mimics the attenuating effect of NAC on ROS. *P <0.05 vs CON; #P <0.05 vs Vehicle/LC.

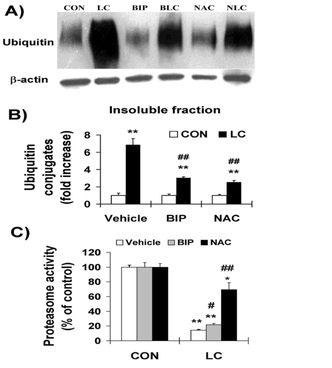

Fig. 3. Iron and ROS participates in the formation of protein aggregation associated with proteasome inhibition. (A+B) Proteasome inhibition-induced protein aggregations are reduced by the iron chelator BIP and the antioxidant NAC. **P <0.01 vs CON; ##P <0.01 vs LC/vehicle. (C) Inhibited proteasome activities are alleviated by application of BIP and NAC. **P <0.01 vs CON; #P <0.05, ##P <0.01 vs LC/vehicle.

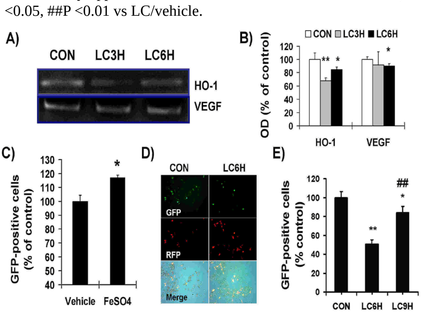

Fig. 4. Effects of UPS impairment on the activity of iron regulatory systems. (A+B) Detecting the activity of HIF/HFE iron regulatory system by RT-PCR. *P <0.05, **P <0.01 vs CON. (C) The live GFP-expression system responses to condition mimicking the increased labile iron. *P <0.05 vs FeSO4 treatment (10mM). (D) GFP-positive cell number is decreased after lactacystin treatment for 6 hr (LC6H) in the live cell reporting system. Green: GFP; Red: RFP. (E) Time course of GFP expression in MES23.5 cells after lactacystin treatment. *P <0.05, **P <0.01 vs CON; ##P <0.01 vs LC6H.

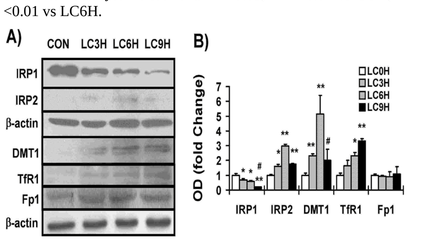

Fig. 5. Iron regulation-related gene expression under the condition of UPS impairment. (A+B) Effects of proteasome inhibition on the gene expression of iron metabolism-related proteins. *P <0.05, **P <0.01 vs CON

RESULTS

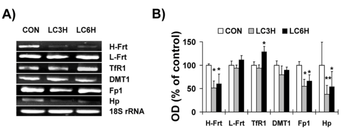

Fig. 6 Changed levels of key iron metabolism-related proteins under conditions of UPS impairment. (A) Western blot showed the time-dependent profiles of protein levels after lactacystin treatment. (B) Diagram showing the change of iron regulatory proteins after lactacystin treatment. *P <0.05, **P <0.01 vs CON; #P < 0.05 vs LC6H.

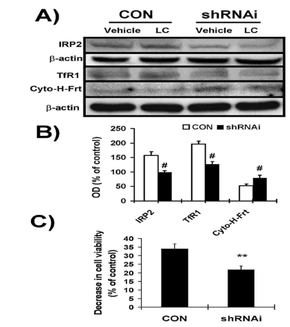

Fig. 7 Involvement of IRP2 in iron misregulation following proteasome inhibition. (**A+B**) Effects of knockdown IRP2 by shRNAi on protein levels of TfR1 and cyto-H-Frt in MES23.5 cells. P <0.05 vs CON. CON, control plasmid for shRNAi; shRNAi, shIRP2 plasmid treatment. LC, lactacystin treated group. (**C**) Knockdown IRP2 partially attenuated the proteasome inhibition-induced DA neuronal injury. *P<0.05, **P<0.01 vs CON.

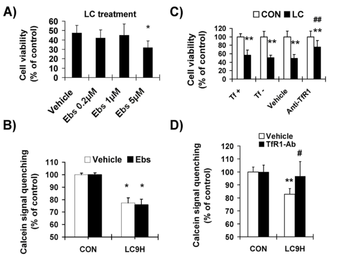

Fig. 8. Role of DMT1 and TfR1 in proteasome inhibition-induced labile iron. (A+B) DMT1 inhibitor Ebs neither protects MES23.5 cell injury (A), nor attenuates the iron increase. (B) Lactacystin treatment. *P <0.05 vs CON. (C) Blocking TfR1 pathway by antibody neutralization protects MES23.5 cell injury. Tf+/-, with or without transferrin in the culture medium. TfR1-Ab, cells treated with TfR1 antibody. **P <0.01 vs CON; ##P <0.01 vs Vehicle/LC. (D) Blocking TfR1 attenuates the calcein signal quenching in MES23.5 cells. **P <0.01 vs CON; #P < 0.05 vs Vehicle/LC.

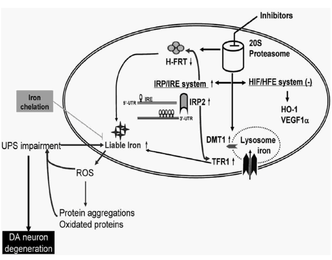

Fig. 8. Neurodegeneration-associated interplay between iron misregulation and UPS impairment involves disturbance in IRP2, TfR1 and H-Frt. Following 20S proteasome inhibitors in MES cells, the IRP/IRE iron regulatory system, but not HIF/HFE system, is disrupted due to IRP2 accumulation. The disruption in IRP2 leads to decrease in H-FRT and increase in TFR1, both of which contributes to the elevated intracellular liable iron level and DA neuron degeneration through generating ROS and enhancing protein aggregations, and in turn exaggerate the existed proteasome dysfunction

CONCLUSIONS

- **We showed that iron misregulation contributes to the UPS impairment-induced neuronal injury through generating ROS and enhancing protein aggregation, which in turn results in worsening of proteasome dysfunction.**
- **Furthermore, our findings suggest that the neurodegeneration-associated interplay between iron misregulation and UPS impairment involves disturbance in IRP2, TfR1 and H-Frt**
- **These results should translate into better understanding of the pathogenesis and treatment of PD.**

ACKNOWLEDGEMENT

Study supported by Diana Helis Henry Medical Research Foundation.