A Mechanistic Study of Proteasome Inhibition-Induced Iron Misregulation in Dopamine Neuron Degeneration

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Abstract
Ubiquitin proteasome system (UPS) impairment and iron misregulation have been implicated in dopamine (DA) neuron degeneration in Parkinson’s disease. As previously shown, proteasome inhibition in a rodent model can cause nigral neuron degeneration accompanied by iron accumulation. To investigate the involvement of iron 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 marked increase in labile iron, reactive oxygen species and ubiquitin-conjugated protein aggregation prior to cell injury. These effects were attenuated by iron chelators or antioxidants. Furthermore, we demonstrated that the iron regulatory protein (IRP)/iron response element system contributed to UPS impairment-mediated DA neuron injury. We documented that IRP2 disruption resulted in an increase in transferrin receptor 1 (TfR1), a decrease in ferritin heavy chain (H-Frt), and eventually cell death. These findings provide insight into the mechanistic interplay between UPS impairment and iron misregulation and suggest that the disturbances in IRP2, TfR1 and H-Frt may contribute to DA neuron degeneration.

Introduction
Parkinson’s disease (PD) is a common neurodegenerative disorder afflicting 1% of the population over the age of 60 [1]. The disease is characterized by the loss of dopamine (DA) neurons in the substantia nigra (SN), and the formation of inclusions called Lewy bodies in the midbrain [2–4]. Recent evidence has shed light upon iron accumulation in the SN as a putative culprit in the pathogenesis of the disease. Although the cause of PD is not completely understood, multiple environmental factors combined with genetic defects probably contribute to the pathogenesis of the disease [1, 5]. Oxidative stress, mitochondrial dysfunction, and aberrant protein degradation have been identified as key pathogenic events associated with DA neurodegeneration in PD [6, 7].

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Recently, it has been proposed that impairment of the ubiquitin proteasome system (UPS) plays an important role in DA neuron degeneration, and iron misregulation has been implicated to contribute to this pathogenic process [3, 6, 8–10]. However, little is known as to the putative interplay between these two pathological events associated with DA neurodegeneration. Accumulating evidence suggests that UPS impairment may disturb iron homeostasis which in turn may further impair UPS function. For example, many proteins involved in iron regulation or iron metabolism are degraded via UPS [11–13]. Accumulation of iron metabolism-related proteins has been observed in the SN of animal models and PD patients [3], and dysfunction of these proteins has been found to be associated with iron accumulation and DA neuron degeneration [3, 14]. Furthermore, it is well documented that excessive iron is potentially neurotoxic because it is associated with increased oxidative stress and generation of reactive oxygen species (ROS), consequently impairing proteasome function and promoting protein aggregates [2, 15, 16]. Interestingly, iron chelation has been shown to have neuroprotective, and in some cases, even neurorestorative effects [17–19].

We previously reported an elevated iron level, accompanied by an increase in the protein levels of iron regulatory protein 2 (IRP2) and divalent metal transporter 1 (DMT1), in the SN of UPS-impaired mice [17]. Our additional study showed that iron chelation not only alleviated neurodegeneration but also restored proteasome activity [17]. Thus, a detailed investigation of the UPS impairment-mediated molecular pathways in a PD cell model is of importance not only for a 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 attempted to explore the mechanism and role of iron misregulation in UPS impairment-induced neurodegeneration using proteasome inhibitor lactacystin treated DAergic cell line MES23.5 by examining the profiles and dynamic changes in iron metabolism/regulation-related genes and proteins and by assessing labile iron level, ROS, protein aggregation, and proteasome activity.

Materials and Methods

Cell Cultures

MES23.5 is a cell line with various phenotypic properties of DA neurons. Originally developed in our laboratory, MES23.5 has been used to study molecular mechanisms underlying DA neuron degeneration and iron misregulation [20–22]. These cells were seeded in dishes, plates or on glass coverslips that were pre-coated with Poly-L-ornithine (10 mg/ml, Sigma, St. Louis, Mo., USA), and cultured in DF12 with 2% SATO supplement, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 2% fetal bovine serum (FBS). Cells were grown to 70% confluence, then we performed transfection using Lipofectamine2000 and Reagent Plus (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer’s instructions. Constructs used in cell transfection included plasmid expressing human ferritin heavy chain under control of tyrosine hydroxylase promoter (pTH-H-Frt, a gift from Professor Julie Andersen, Buck Institute of Age Research, USA), or plasmid p5'-UTR-IRE-GFP-NLS (a gift from Professor Michael Kiebler, Medical University of Vienna, Austria). Cell transfectants were then grown for additional 36 or 48 h before further analysis. For establishing a stable cell line expressing human H-Frt, the MES23.5 cells transfected with plH-H-Frt were incubated in medium with G418 (50 μg/ml) for 14 days. After that the single colony was cultured in medium with G418.

Cell Treatments

For this study, MES23.5 cells were grown to 80% confluence and then treated with the irreversible proteasome inhibitor lactacystin (5 μM; AG Scientific Inc., San Diego, Calif., USA) after pretreatment respectively for 1 h with vehicle (LC), or iron chelators deferoxamine (DFO, Sigma) or bipyridyl (BIP, Sigma), antioxidant N-acetyl cysteine (NAC, Sigma), or DMT1 inhibitor Ebselen (Ebs, Sigma) at the indicated concentrations, or anti-transferrin receptor 1 antibody (anti-TfR1, 1:400 dilution; Alpha Diagnostics Intl. Inc., San Antonio, Tex., USA). Some of the cultures were treated with proteasome inhibitor MG-132 (AG Scientific Inc.) at 2 μM for 9 h.

Cell Viability Assay

MES23.5 cells were seeded on a 96-well plate, grown for 24 h, and then pretreated respectively with vehicle, DFO, NAC, Ebs or anti-TfR1 antibody followed by exposure to lactacystin (5 μM) for 12 or 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT, 0.5 mg/ml; Sigma) was used to detect the viability according to previous methods [23].

Detection of Labile Iron and ROS

Calcein fluorescence signal can be quenched by labile iron [20]. Therefore intracellular labile iron was measured by calcein fluorescence quenching according to previously described methods [20]. ROS were detected by using CM-H2DCFDA (Invitrogen) according to the manufacturer’s instructions with a few modifications. Briefly, MES23.5 cells were seeded on a 96-well plate. After lactacystin treatment, cells were washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (1.6% NaCl, 0.074% KCl, 0.027% Na2HPO4·2H2O, 0.2% dextrose, and 1% HEPES), and then incubated with calcein-AM (2 μM) or CM-H2DCFDA (10 μM) in HEBS for 30 min at 37°C. CMRA (1 μM, a gift from Dr. Wang Jun, MD Anderson Cancer Center) was used as internal reference. Fluorescence intensity was recorded at a series of time points after lactacystin treatment (0, 3, 6, and 9 h) by using a microplate fluorometer (Limit 1, ex/em 490/522 nm; Limit 2, ex/em 548/576 nm). Ferrous sulfate solution (1 mM) was added in culture medium as a positive control.
Western Blot Assay
At the scheduled time points following treatments, proteins of MES23.5 cells were harvested according to previous descriptions [23, 24]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transblot were performed according to previous methods [23, 24]. The probed primary antibodies included: anti-IRP1, anti-IRP-2, anti-TfR1, anti-DMT1, anti-ferroportin 1 (anti-Fp1, 1: 1000; Alpha Diagnostics Intl. Inc.), anti-ubiquitin (anti-IRP1, 1: 1000; Alpha Diagnostics Intl. Inc.), anti-Thioredoxin (1: 4000, Sigma) were set as the internal standard. Signals of the probed primary antibodies included: anti-IRP1, anti-IRP-2, anti-TfR1, anti-DMT1, anti-ferroportin 1 (anti-Fp1, 1: 1000; Alpha Diagnostics Intl. Inc.), anti-ubiquitin (anti-IRP1, 1: 1000; Alpha Diagnostics Intl. Inc.), anti-Thioredoxin (1: 4000, Sigma) were set as the internal standard. Signals of the protein bands were detected with SuperSignal West Dura Substrate kit (Thermo Scientific).

Proteasome Activity Assay
MES23.5 cells were lysed in an ice-cold assay buffer as previously described [17]. The chymotrypsin-like activity was detected by using a 20s proteasome activity kit (Chemicon International Inc., USA) according to the manufacturer’s instructions and was normalized by protein concentration.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction
RNA was isolated from cells using Trizol Reagent according to our previous protocols [23]. The first strain was synthesized with a Superscript kit (Invitrogen). Iron regulation-related genes, including H-Frt, ferritin light chain (L-Frt), TfR1, DMT1, Fp1, hephaestin (Hp), heme oxygenase 1 (HO-1) and vascular endothelial growth factor 1 (VEGF1) (table 1) were amplified using a Qiagen Hotstar polymerase chain reaction (PCR) kit. 18S rRNA was set as the internal standard, and semi-quantification was performed with Quantity-One software according to previous methods [23]. Briefly, PCR products were separated on agarose gels. Ethidium bromide-labelled intensity of targeted bands was examined by Quantity-One software and normalized by their internal standard and their control respectively.

Detection of IRP/Iron Response Element System Activity in Living Cells
To determine the IRP/iron response element (IRE) binding activity under conditions of UPS dysfunction, MES23.5 cells were seeded on glass coverslips at a density of 5 × 10^4 per well and grown for 24 h, then transfected with a plasmid that expressed a nuclear location sequence (NLS)-fused green fluorescent protein (GFP) under control of a 233-bp H-Frt promoter containing IRE (p5’UTR-IRE-GFP-NLS) as previously mentioned [25]. A plasmid expressing red fluorescence protein (pds-RFP-N1, a gift from Dr. Yang Weiwei, MD Anderson Cancer Center) was co-transfected as an internal standard and their control respectively.

Iron and UPS Impairment in DA Neuron Degeneration

Table 1. Primers for RT-PCR

| Primer name | Sequence | Access no. | Cycle number |
|-------------|----------|------------|--------------|
| HO-1 Forward | 5’-AAACCAGCAGCCCCAAATCCTG-3’ | NM_012580.2 | 36 |
| Reverse: 5’-ACACAGAAGTTAGAGACAAAGTACC-3’ | | | |
| VEGF1α Forward | 5’-GGGAGCCCTCAGGACATGGCCTTT-3’ | NM_031836.2 | 36 |
| Reverse: 5’-GGGAGCCCTCAGGACATGGCCTTT-3’ | | | |
| H-Frt Forward | 5’-CCCACCTTTGATCTATGGAGACG-3’ | NM_012848.4 | 32 |
| Reverse: 5’-CACGCTGACATGCACTGAGCT-3’ | | | |
| L-Frt Forward | 5’-TGACACTCTCCAGGTAGTG-3’ | NM_022500.3 | 36 |
| Reverse: 5’-TCAAGAGATACCTGCCCGAG-3’ | | | |
| TfR1 Forward | 5’-GCCGTGCTACTCTAGACTAACC-3’ | NM_022712.1 | 36 |
| Reverse: 5’-TTCCAAAATGTCAAACCAGAGG-3’ | | | |
| DMT1 Forward | 5’-AGTTTGTCACTGGAGATTCTCTG-3’ | NM_013173 | 38 |
| Reverse: 5’-CTAACCAGCTGAGCAACCAC-3’ | | | |
| Fp1 Forward | 5’-CTTGTGTGATGATCCCTCGTGTTCAT-3’ | NM_133315.2 | 37 |
| Reverse: 5’-CAAAGGACAAAGAAGCGATTTGAC-3’ | | | |
| Hp Forward | 5’-GATGTGTGTCTACTGCTATGGATCTGCG-3’ | NM_133304.1 | 37 |
| Reverse: 5’-GAAGGATGCGCATATGGCTAACTGCGA-3’ | | | |
| 18S rRNA Forward | 5’-CGG CTA CCA CAT CCA AGG GAA-3’ | M11188.1 | 28 |
| Reverse: 5’-GCT GGA ATT ACC GCG GCT-3’ | | | |

HO-1 = Heme oxygenase 1; VEGF1α = vascular endothelial growth factor 1α; H-Frt = ferritin heavy chain; L-Frt = ferritin light chain; TfR1 = transferrin receptor 1; DMT1 = divalent metal transporter type 1; Fp1 = ferroportin 1; Hp = hephaestin.
was fused at the N-terminus of GFP, which makes it possible to examine the GFP level by counting cells with GFP-positive nuclei under an immunofluorescence microscope. FeSO$_4$ (10 mM) was set as positive control to test the efficacy of this system.

**Lentivirus ShRNA Vector-Based Gene Knockdown**

To investigate the role of DMT1 and IRP2 in UPS impairment-induced DA cell injury, lentivirus-based shRNA interference was used to knock down DMT1 and IRP2 in MES23.5 cells. A pair of synthesized oligo containing restriction enzymatic cleavage sites of Bam I at the 5'-terminus and EcoR I at the 3'-terminus (table 2) was subcloned into a pSIH-H1-copGFP vector (SBI System Biosciences, Calif., USA). The construct (pSIH-DMT1) was verified either by PCR or sequencing, and then transfected to HEK293T cells with pPACK packaging plasmid mix (SBI System Biosciences) according to the manufacturer's instructions. Transfectants were maintained in DMEM with 10% FBS and antibiotics (Gibico, Carlsbad, Calif., USA) at 37 °C with 5% CO$_2$. Viral particles in the medium were collected and MES23.5 cells were then exposed to a mixture of the medium containing viral particles and polybrene (50 μg/ml, Sigma) for 12 h and then maintained in DF12 supplemented with 1! SATO, 2% FBS and antibiotics for 60 h before treatment.

**Immunoprecipitation**

MES23.5 cells were scraped and lysed in an ice-cold lysis buffer (1.5 mM MgCl$_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM ethylene glycol tetraacetic acid, 1 mM Na$_3$VO$_4$, 5 mM sodium fluoride, 0.4% IGEPAL, in 10 mM HEPES pH 8.0) with 1 × protease inhibitor cocktail (Roche). After a brief centrifugation (15,000 g, 3 min) at 4°C, the supernatants were harvested as cytosolic fraction. The lysates were quantified and the same amount (150 μg) was incubated with 25% protein G-Sepharose 4 Fast Flow slurs (20 μl, GE Healthcare, UK) at 4°C for 30 min. After a brief centrifugation, the supernatant was washed with radioimmunoprecipitation assay buffer, re-suspended in sample buffer, and then subjected to SDS-PAGE and immunoblot.

**Data Analysis**

The data collected from experimental groups were normalized by their control and presented as mean ± SEM. One-way analysis of variance (ANOVA) and post hoc least significant difference (LSD) was used to examine the difference between experimental groups. p < 0.05 was set as significant. All experiments were replicated at least on two independent cell cultures.

**Results**

**Labile Iron Is Increased following Proteasome Inhibition and DA Cell Injury**

An initial experiment was set up to examine the labile iron level by calcein signal quenching at different time points after addition of the proteasome inhibitor lactacystin in MES23.5 cells. We found that labile iron elevation started to show at 6 h and peaked at 9 h after lactacystin treatment (fig. 1A; p < 0.01 vs. CON, n = 7; p < 0.05 vs. LC6H, n = 6). In the positive control group, FeSO$_4$ treatment for 9 h quenched calcein signal by 27% (fig. 1A; p < 0.01, n = 6), suggesting that proteasome inhibition caused an early increase in labile iron level in MES23.5 cells. We then examined the cell viability and labile iron level following pretreatment with DFO (1 mM). We found that DFO prevented the lactacystin-induced cell injury by 81% (fig. 1B; p < 0.05, n = 4) and alleviated the lactacystin-induced labile iron level at 9 h by 75% (fig. 1C; p < 0.01, n = 3), suggesting a contribution of the UPS impairment-induced labile iron in cell injury. Change in labile iron...
level was reproduced by another proteasome inhibitor, MG-132 (fig. 1D; n = 8), indicating iron misregulation specifically resulted from proteasome inhibition.

**UPS Impairment-Induced Labile Iron and ROS Exaggerate Protein Aggregation and Proteasome Impairment**

To determine the role of UPS impairment-induced labile iron in MES23.5 cell injury, we detected ROS production at an early stage of lactacystin treatment. Lactacystin treatment induced a significant increase in ROS at 9 h (fig. 2A; p < 0.01, n = 7). Pretreatment of NAC (10 mM) prevented MES cell loss by 36% (fig. 2B, C; p < 0.05, n = 5) and blocked the labile iron level at 9 h by 53% (fig. 2D; p < 0.05, n = 4). A similar blockade effect of NAC on the lactacystin-induced ROS production was shown at 9 h (fig. 2E; p < 0.05, n = 4). Moreover, application of BIP (500 μM) mimicked the effects of NAC on ROS production (fig. 2F; p < 0.05, n = 4). These results suggest an interaction between ROS and labile iron associated with UPS impairment-induced DA neuron injury.

It is well known that labile iron can generate ROS and promote protein aggregation. Both of these effects impair UPS function [15, 16, 19]. Therefore, we tested whether a vicious circle might be formed by labile iron-related ROS in our cell model. We found that lactacystin treatment caused a marked increase in ubiquitin conjugates in the insoluble fraction at the early stage, which could be reduced by pretreatment with either BIP (500 μM, BLC) or NAC (10 mM, NLC) (fig. 3A, B; p < 0.01, n = 3). Furthermore, pretreatment with BIP, or NAC significantly restored the inhibition of lactacystin treatment on the proteasome activity (fig. 3C; p < 0.05 in BLC and p < 0.01 in NLC, n = 3). These results suggest that iron misregulation aggravates UPS impairment by generating ROS and promoting protein aggregation.

**Proteasome Inhibition Disturbs the IRP/IRE Regulatory System**

Iron homeostasis is primarily maintained by the IRP/IRE system and the hypoxia-inducible factor (HIF)/HIF-responsive elements (HRE) system [2, 26, 27]. We examined the activities of these 2 regulatory systems following lactacystin treatment. HO-1 and VEGF1α, two genes under regulation of the HIF/IRE system, were used as indicators. We found a slight to moderate decrease in these two transcripts following lactacystin treatment for 3–6 h (fig. 4A, B; p < 0.05, n = 3), suggesting the HIF/IRE system was not severely affected in the early stages of UPS impairment.

Next, we tested the activity of the IRP/IRE system in live cells by transient transfection of the plasmid p5′UTR-IRE-GFP-NLS in MES23.5 cells as previously described [25]. FeSO₄ treatment (10 μM for 9 h, positive
control) to disturb IRP/IRE binding caused a significant increase in GFP-positive cells (fig. 4C; p < 0.05, n = 3). After lactacystin treatment for 6 h, we detected a 49% decrease in GFP-positive cells (fig. 4D; n = 8), which was partially recovered at 9 h (fig. 4D, E; p < 0.01 vs. LC6H, n = 11).

We then examined the expression level of iron metabolism-related genes following lactacystin treatment by semi-RT-PCR, showing decreased mRNA levels in genes encoding the iron chelation protein H-Frt but not in L-Frt (fig. 5A; n = 3). The transcript of iron uptake protein TfR1 was increased at 6 h, whereas that of DMT1 remained unchanged (fig. 5B). The mRNA levels of iron export proteins Fp1 and Hp were significantly decreased in the early stages of UPS impairment (fig. 5B; p < 0.05). These results suggest a perturbation in the IRP/IRE regulatory system and iron regulation-related gene expression following UPS impairment.

**H-Frt and TfR1 Are Involved in the Iron Misregulation following Proteasome Inhibition**

To examine the protein levels of several iron regulation-related molecules after lactacystin treatment, we conducted a Western blot assay on IRP1, IRP2, DMT1, TfR1, Fp1 and H-Frt. We found an increase in the protein levels of IRP2, DMT1 and TfR1 in the early stages of UPS impairment (fig. 6A, B; n = 3). While IRP2 and TfR1 remained at higher levels, DMT1 levels decreased at 9 h (fig. 6A, B). Fp1 remained unchanged but IRP1 decreased during the observed period (fig. 6A, B).
Fig. 3. Iron and ROS participate in the formation of protein aggregation associated with proteasome inhibition. **A, B** Proteasome inhibition-induced protein aggregations are reduced by the iron chelator BIP (500 μM) and the antioxidant NAC (10 mM). *p < 0.01 versus CON; **p < 0.01 versus LC/vehicle, n = 3. C Inhibited proteasome activities are alleviated by application of BIP and NAC. *p < 0.05; **p < 0.01 versus CON; *p < 0.05, **p < 0.01 versus LC/vehicle, n = 3. Data are presented as mean ± SEM. BLC = BIP plus LC; NLC = NAC plus LC.

Fig. 4. Effects of UPS impairment on the activity of iron regulatory systems. **A, B** Detecting the activity of the HIF/HFE iron regulatory system under conditions of UPS impairment by RT-PCR. *p < 0.05, **p < 0.01 versus CON, n = 3. OD = Optic density. **C** The live GFP expression system responses to a condition mimicking increased labile iron. *p < 0.05 versus FeSO₄ treatment (10 μM), n = 3. **D** GFP-positive cell number is decreased after lactacystin treatment for 6 h (LC6H) in the live cell reporting system. RFP = Red fluorescence protein. Green: GFP; Red: RFP. Colors refer to the online version only. **E** Time course of GFP expression in MES23.5 cells after lactacystin treatment. *p < 0.05, **p < 0.01 versus CON; *p < 0.01 versus LC6H group, n = 8. Data are presented as mean ± SEM.
Next, we examined the role of DMT1 and TfR1 in the iron misregulation after proteasome inhibition. Initially, we applied a newly identified DMT1 inhibitor, Ebs [28], in the lactacystin-treated MES23.5 cells. Unexpectedly, Ebs neither protected against the lactacystin-induced cell injury (fig. 7A; 0.2–5 μM, n = 6) nor affected the labile iron level (fig. 7B; 1 μM, n = 8) and ROS production (data not shown). Knockdown of the DMT1 gene by lentivirus-based shRNAi also failed to protect against UPS impairment-induced MES23.5 cell injury (data not shown), suggesting that other mechanisms might be involved. Using TfR1 antibody binding to the extracellular domain to block the TfR1-mediated iron uptake [29] in the lactacystin-treated MES23.5 cells, we found that anti-TfR1 antibody significantly alleviated cell injury by 53% (fig. 7C; * p < 0.05 versus CON, n = 3) and reversed labile iron level by 81% at 9 h after lactacystin treatment (fig. 7D; * p < 0.05 versus LC3H, n = 4), suggesting a role of TfR1 in iron misregulation and cell injury following proteasome inhibition.

Previously, we reported that H-Frt has protective effects against nigral DA neuron loss under the condition of oxidative stress and UPS impairment in vivo [17]. Here,
we measured the protein level of cytoplasmic H-Frt using immunoprecipitation and found that lactacystin treatment caused a 51% decrease of H-Frt (fig. 8A, B; p < 0.05, n = 3). Using a MES23.5 cell line that overexpresses human H-Frt, we found a 74% reduction in labile iron level (fig. 8C; p < 0.05, n = 11) and a 25% increase in cell viability (fig. 8D; p < 0.05, n = 7) as compared with lactacystin-treated mock MES23.5 cells, indicating that disturbed levels of H-Frt contribute to the iron misregulation and cell injury following proteasome inhibition.

**Altered IRP2 Level Mediates Iron Misregulation under the Condition of UPS Impairment**

Finally, we determined whether the altered level of IRP2 is involved in the changes in Tfr1 and cyto-H-Frt as well as cell viability following lactacystin treatment. We
applied IRP2 shRNAi to knock down IRP2 expression which was reduced by 40% in 72 h (fig. 9A; p < 0.05, n = 5). The pretreatment with IRP2 shRNAi attenuated the lactacystin-induced increase in TfR1 and IRP2 by 38 and 39%, and the lactacystin-induced decrease in cyto-H-Frt by 49%, respectively (fig. 9A, B; all p < 0.01, n = 3). Furthermore, we documented that cell viability was increased by 36% in the IRP2 shRNAi-pretreated MES23.5 cells as compared with controls after lactacystin treatment (shRNAi vs. control, p < 0.01; fig. 9C, n = 6). These experiments suggest IRP2 underlies the TfR1/H-Frt-related iron misregulation and cell injury under the condition of UPS impairment.

**Discussion**

Both UPS impairment and abnormal iron deposition have been reported in the aging process [30] and in several neurological diseases, including PD [1, 7]. But the association between UPS impairment and iron misregulation has not been determined. In this study, we have shown for the first time that UPS impairment leads to iron misregulation via TfR1, H-Frt and IRP2. Furthermore, we demonstrated that iron misregulation contributes to UPS impairment-induced cell injury through the generation of ROS and protein aggregation. These findings may add to our understanding of the pathogenesis of PD, and imply that restoration of proteasome activity by iron chelation may slow or prevent neurodegeneration and thus might be a potential therapeutics target in PD.

Iron is a crucial metal ion needed for multiple brain functions including neurotransmitter metabolism, DNA synthesis, cell cycle regulation, gene expression, mitochondrial electron transport and myelination [2, 19]. On the other hand, elevated iron has been found in the nigral DA neurons in PD [31–33]. This is supported by the recent report that high levels of iron are present in the SN of PD patients even before the appearance of clinical motor symptoms [34]. Furthermore, mutations in genes related to iron metabolism and regulation have been documented in many neurological diseases known collectively as neurodegeneration with brain iron accumulation [34]. It is well known that iron-related toxicity is mainly medi-
Increased labile iron can cause oxidative stress in the SN of PD while total iron levels remain unchanged [35]. Studies have shown that increased labile iron causes oxidative stress and increases neuronal vulnerability by aggravating the toxicity of other putative environmental or endogenous pathogens [14–16], leading to preferential DA neuron injury in PD. Recently, we [17] and Vernon and colleagues [36] reported elevated nigral iron levels in a UPS impaired rodent model. But these observations did not define whether an increase in iron level is directly from nigral DA neurons or secondarily from non-neuronal cells in SN. In the DA cell line treated with proteasome inhibitors, we documented that proteasome inhibition can lead to an early increase in labile iron. As Hasinoff [37] pointed out, the calcein quenching method has a limited capacity to evaluate labile iron levels under conditions of oxidative stress. In our study, we found that the iron chelator DFO can effectively attenuate the labile iron elevation, implying that proteasome inhibition can directly cause iron misregulation in DA neurons. Furthermore, we demonstrated that the increased labile iron level in turn aggravates proteasome inhibition through generation of ROS and promotion of protein aggregation; while restoring proteasome activity seems to enhance removal of protein aggregates and attenuation of oxidative stress [38].

UPS impairment may result in increased ROS production. In our study, we documented significantly higher levels of ROS in an early stage of proteasome inhibition, which is in agreement with other findings in lactacystin-treated cell models of PD [39]. In addition, ROS overproduction induced by proteasome inhibition together with mitochondrial dysfunction inhibited proteasome function recovery and mediated the persistent neurotoxicity [40]. In neuronal cells, two iron-regulatory systems, including the IRP/IRE and HIF/HRE systems, sense and control the intracellular iron level by different mechanisms [2, 27, 41]. HO-1 and VEGF1α are regulated by the
HIF/HFE system [27] and are degraded by UPS [42]. Our results suggest that the HIF/HFE system may not participate in the increased labile iron following proteasome inhibition, but it may not be safe to completely rule out the role of HIF/HFE. In contrast, the interaction of the IRP/IRE system seems to increase as shown by the 5′-IRE-GFP detection system. Interestingly, the reduced GFP translation is partially reversed after lactacystin treatment for 6 h, which correlates with the increased labile iron level and may cause disturbance in IRP/IRE binding [2, 26, 41]. Initial decrease in GFP-positive cells in the lactacystin-treated cells may be attributed to elevated IRP2 by blocking its translation. The increase in IRP/IRE binding is supported by the increased TfR1 mRNA since binding of IRP to the multiple IREs at the 3′-UTR significantly increases the stability of this transcript. In addition, we showed that increased IRP2 is involved in cell injury; a similar result has been seen in a 6-OHDA-induced DA neuron degeneration model [22]. The IRP/IRE system has been gaining more attention because modulation of the IRP/IRE interaction may play a role in protective effects [22]. Therefore, IRP might be a potential target for neuroprotection against iron-related neurotoxicity [43].

A sustained increase in TfR1 and a decrease in cytoplasmic H-Frt are involved in the UPS impairment-induced iron misregulation and cell injury in the present study. Prior research already provided evidence that TfR1-mediated iron intake contributes to DA neurodegeneration following glutathione depletion [10] and that 1-methyl-4-phenylpyridinium (MPP+) intoxication can be attenuated by TfR1 antibody neutralization [29]. However, TfR1 expression is relatively low in nigral DA neurons and is not significantly altered in the remaining nigral DA neurons in PD [2, 26]. One explanation for this is that nigral DA neurons with higher levels of TfR1 might be more vulnerable and may degenerate earlier. It seems that the Tf in the FBS is enough for MES cells to form a Tf/iron/TfR complex during the uptake of iron, since our results indicate that deprivation of Tf in SATO supplement (Tf−) acts similarly to the Tf+ group.

H-Frt is a robust endogenous iron chelator [44]. In human nigral neurons the level of H-Frt is relatively low [2, 26], and in PD the Frt level remained unchanged in the SN where the total iron level is increased [45]. H-Frt is degraded via both UPS and the autophagy-lysosome pathway [44, 46]. In our study, we documented a decreased cyto-H-Frt, and blocking the lysosome pathway by leupeptin and NH₄Cl does not affect the iron misregulation and cell injury (data not shown), suggesting that the lysosome pathway may not cause the decrease in cytoplasmic H-Frt.

In this study, DMT1 was found to accumulate under the condition of UPS impairment, consistent with our previous findings in the SN of UPS-impaired mice [17]. DMT1 has been associated with DA neurodegeneration in certain contexts [3, 22] and the involvement of DMT1 in iron-mediated neurodegeneration has been documented in experiments using a 6-OHDA-intoxicated MES23.5 cell model [22]. However, we did not observe a close association of DMT1 with iron misregulation and cell injury in this study. Further investigation on a primary mesencephalic cellular model and studies on the interaction between different isoforms of DMT1 may help us define the exact mechanism. Whereas blocking DMT1 has no significant effect, TfR1 antibodies significantly prevent iron misregulation and cell injury in our study. It seems that some molecular mechanisms other than DMT1 may facilitate iron translocation from the endosome/lysosome to the cytoplasm, underlying the TfR1-mediated iron misregulation in our study.

In conclusion, we have demonstrated that iron misregulation contributes to UPS impairment-induced neuronal injury through generating ROS and enhancing protein aggregations, which in turn exaggerates proteasome dysfunction (fig. 10). Our study may provide evidence showing that the interaction between iron misregulation and UPS impairment, as well as the IRP2, TfR1 and H-Frt disturbance may contribute to DA neuron degeneration. These findings may improve our understanding of the neuropathogenesis of PD.

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References

1 Dawson TM, Dawson VL: Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003; 302:819–822.

2 Rhodes SL, Ritz B: Genetics of iron regulation and the possible role of iron in Parkinson's disease. Neurobiol Dis 2008;32:183–195.

3 Salazar J, Mena N, Hunot S, Prigent A, Alvarez-Fischer D, Arredondo M, Duyskaerts C, Szadovitch Y, Zhao L, Garrick LM, Nunez MT, Garrick MD, Raisman-Vozari R, Hirsh EC: Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease. Proc Natl Acad Sci USA 2008;105:18578–18583.

4 Crichton RR, Dexter DT, Ward RJ: Brain iron metabolism and its perturbation in neurodegenerative diseases. J Neural Transm 2010;118:301–304.

5 Yacoubian TA, Standaert DG: Targets for neuroprotection in Parkinson's disease. Biochim Biophys Acta 2009;1792:676–687.

6 McNaught KS, Olanow CW: Protein aggre-nation 2006; 27: 530–545.

7 Levy OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

8 Levy OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

9 Salazar J, Mena N, Hunot S, Prigent A, Alvarez-Fischer D, Arredondo M, Duyskaerts C, Szadovitch Y, Zhao L, Garrick LM, Nunez MT, Garrick MD, Raisman-Vozari R, Hirsh EC: Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease. Proc Natl Acad Sci USA 2008;105:18578–18583.

10 Crichton RR, Dexter DT, Ward RJ: Brain iron metabolism and its perturbation in neurodegenerative diseases. J Neural Transm 2010;118:301–304.

11 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

12 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

13 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

14 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

15 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

16 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

17 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

18 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

19 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

20 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

21 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

22 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

23 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

24 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

25 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

26 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

27 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

28 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

29 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

30 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

31 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

32 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

33 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

34 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

35 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

36 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

37 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

38 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

39 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

40 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

41 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

42 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

43 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

44 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.

45 Layer OA, Malagelada C, Greene LA: Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 2009;14:478–500.
39 Keller JN, Huang FF, Dimayuga ER, Maragos WF: Dopamine induces proteasome inhibition in neural PC12 cell line. Free Radic Biol Med 2000;29:1037–1042.

40 Papa L, Rockwell P: Persistent mitochondrial dysfunction and oxidative stress hinder neuronal cell recovery from reversible proteasome inhibition. Apoptosis 2008;13:588–599.

41 Hentze MW, Muckenthaler MU, Galy B, Camaschella C: Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24–38.

42 Kaluz S, Kaluzová M, Stanbridge EJ: Does inhibition of degradation of hypoxia-inducible factor (HIF) alpha always lead to activation of HIF? Lessons learnt from the effect of proteasomal inhibition on HIF activity. J Cell Biochem 2008;104:536–544.

43 Recalcati S, Minotti G, Cairo G: Iron regulatory proteins: from molecular mechanisms to drug development. Antioxid Redox Signal 2010;13:1593–1616.

44 Arosio P, Ingrassia R, Cavadini P: Ferritins: a family of molecules for iron storage antioxidation and more. Biochim Biophys Acta 2009;1790:589–599.

45 Dexter DT, Jenner P, Schapira AH, Marsden CD: Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. Ann Neurol 1992;32:S94–S100.

46 Zhang YH, Mikhael M, Xu D, Li Y, Shan SL, Ning B, Li W, Nie GJ, Zhao YL, Ponka P: Lysosomal proteolysis is the primary degradation pathway for cytosolic ferritin and cytosolic ferritin degradation is necessary for iron exit. Antioxid Redox Signal 2010;13:999–1009.