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Ferroportin1 and Hephaestin Overexpression Attenuate Iron-Induced Oxidative Stress in MES23.5 Dopaminergic Cells

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ABSTRACT

Elevated iron was found in the substantia nigra (SN) of patients with Parkinson's disease (PD). Our previous in vivo experiments suggested that decreased ferroportin1 (FPN1) and hephaestin (HP) expression might account for the cellular iron accumulation and resulting dopaminergic neurons loss in the SN of PD animal models. In the present study, we investigated whether increased FPN1 and/or HP expression could attenuate iron-induced oxidative stress in the dopaminergic MES23.5 cell line. We generated MES23.5 cells with stable overexpression of FPN1 and/or HP. Our study showed that overexpression of FPN1 and/or HP increased iron efflux, lowered cellular iron level, suppressed reactive oxygen species production, and restored mitochondrial transmembrane potential, similar to the effects seen for the iron chelator deferoxamine. These results suggest that FPN1 and/or HP might directly contribute to iron efflux process from neurons in conditions of overexpression, thus prevent cellular iron accumulation and eventually protect cells from iron-induced oxidative stress. J. Cell. Biochem. 110: 1063–1072, 2010.

KEY WORDS: FERROPORTIN1; HEPHAESTIN; IRON; IRON CHELATOR; OXIDATIVE STRESS; PARKINSON'S DISEASE

Abnormally high levels of iron have been observed in the substantia nigra (SN) of patients with Parkinson's disease (PD), indicating that cellular iron balance is disrupted [Curtis et al., 2001; Perry et al., 2002; Gotz et al., 2004; Zecca et al., 2004; Berg and Hochstrasser, 2006; Oakley et al., 2007; Martin et al., 2008]. In our previous studies, we found that divalent metal transporter 1 (DMT1) up-regulation might be involved in the iron accumulation of PD animal and cell models [Jiang et al., 2003; Song et al., 2007; Xu et al., 2008; Zhang et al., 2009]. For maintenance of a balanced iron homeostasis, the cellular iron efflux, as well as iron influx must be tightly regulated. In recent years, the study of iron metabolism has been revitalized following the cloning and characterization of some new iron transport related proteins, including ferroportin1 (FPN1) and hephaestin (HP).

FPN1 (also known as MTP1, IREG 1, or SLC11A3) was a multiple transmembrane domain protein identified in several cell types to be involved in the export of cellular iron [Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000]. It was the only channel for cellular iron efflux [Ganz, 2005]. FPN1 functions in placental materno-fetal iron transfer, intestinal iron absorption and iron release from reticuloendothelial macrophages [Devalia et al., 2002]. HP, a multicopper ferroxidase, is a membrane-bound ceruloplasmin (CP) homologue also necessary for iron export from enterocytes and possibly necessary for iron incorporation into transferrin [Vulpe et al., 1999]. HP itself, as an enzyme with only one transmembrane domain, is unlikely to be a membrane iron transporter [Vulpe et al., 1999; Anderson et al., 2002a]. In the presence of an iron transporter (FPN1) and a ferroxidase (HP), the newly released ferrous iron could be oxidized to its ferric form, allowing binding to transferrin. This defines a key role for FPN1 and HP co-operation in physiological iron export.

How FPN1 and HP contribute to cellular iron homeostasis of CNS was still not fully understood. Our previous study in a rat model of

Abbreviations used: CP, ceruloplasmin; DFO, deferoxamine; DMT1, divalent metal transporter 1; DMEM, Dulbecco's modified Eagle's medium; FAC, ferric ammonium citrate; FPN1, ferroportin1; HBS, HEPES-buffered saline; HP, hephaestin; PD, Parkinson's disease; SN, substantia nigra; TfR1, transferrin receptor 1.

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PD demonstrated that both FPN1 and HP were expressed on the neurons as well as glia in the SN and down-regulations of FPN1 and HP might account for nigral iron deposit and thus dopaminergic neurons death [Wang et al., 2007]. Due to the suggested involvement of FPN1 and HP in cellular iron accumulation in PD, in the present study, we stably overexpressed FPN1 and/or HP using expression vector construction in MES23.5 cells and determined how this influenced iron efflux, as well as cellular iron level and iron induced oxidative stress. The MES23.5 dopaminergic cell line was chosen as a model for dopaminergic neurons since it exhibits several properties similar to the primary neurons originated in the SN [Crawford et al., 1992]. The results showed that both FPN1 and HP increase iron efflux in conditions of overexpression, transport more iron outside, and thus protect cells from iron induced oxidative stress, similarly to the protective effects of iron chelator deferoxamine (DFO). This study suggests that FPN1 and HP could serve as targets to attenuate intracellular iron accumulation in iron-related neurodegenerative diseases.

MATERIALS AND METHODS

MATERIALS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis. MO). The primary antibodies against FPN1 or HP were from the Alpha Diagnostic (ADI, San Antonio, TX). Dulbecco's modified Eagle's medium (DMEM)/F12 was from Gibco (Grand Island, NY). *Bam*HI, *Xba*I, *Eco*RI, *Xho*I, and PMD18-T simple vector were from TaKaRa (TaKaRa Biotechnology, Dalian, China). Calcein-AM was from Molecular Probes (Molecular Probes, Inc., Carlsbad, CA). All other chemicals and regents were of the highest grade available from local commercial sources.

CELL CULTURE

Rat mesencephalic dopaminergic cells (MES23.5 cells) were obtained by courtesy of Dr. Weidong Le (Department of Neurology, Baylor College of Medicine, Houston, USA). MES23.5 cells were cultured in DMEM/F12 growth medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L glutamine and Sato's chemically defined medium to a final concentration of 5 μg/ml insulin, 5 μg/ml transferrin, 48.6 μg/ml pyruvic acid, 6.3 ng/ml progesterone, 5 ng/ml sodium selenite, 4 μg/ml putrescine at 37°C, in a humid 5% CO_2, 95% air environment. For experiments, cells were sub-cultured twice a week when grown to 70–80% confluence and seeded at a density of $10^5/cm^2$ in polylysine-precoated plastic flasks or plates.

CONSTRUCTION OF RECOMBINANT EXPRESSION VECTORS PIRES-FPN1 AND PIRES-HP-FPN1

The full length rat FPN1 sequence was inserted into a stable mammalian vector pIRES (a kindly gift from Dr. Xiaomin Wang, Neuroscience Research Institute, Peking University, Beijing, China) containing two multiple cloning sites, the markers of ampR for selection in bacterial cells, neoR for selection and a CMV promoter for expression in mammalian cells. For cloning the full length FPN1 cDNA, nested PCR was conducted (two sets of primers were as follows: external forward 5′-GAGAGCAGGCTTAGGGTC-3′, external reverse 5′-AGTGCAGGCATTTATTGG-3′; internal forward 5′-AGGCTTTAGCTTTCCAACT-3′, internal reverse 5′ AGGGGCCACAG-CTAAACT3′); the 1,989-bp PCR fragment was cut with restriction enzymes *Bam*HI and *Xba*I and then were subsequently inserted into the PMD18-T simple vector for cloning and sequencing. The digested fragment was inserted into pIRES vector at the second multiple cloning site of to form the new construct donated pIRES-FPN1.

For construction of recombinant vector pIRES-HP-FPN1, the full length mouse HP cDNA was prepared from the plasmid pcDNA3.1-HP (a kindly gift from Dr. Jerry Kaplan, Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, USA) by PCR, which contained restriction endonuclease sites *Eco*RI and *Xho*I. Then, the purified HP cDNA (3,516 bp) was inserted into plasmid pIRES-FPN1 at the first multiple cloning site and formed the construct donated pIRES-HP-FPN1.

TRANSFECTION AND SELECTION OF STABLE RECOMBINANT CELL LINES

MES23.5 cells were seeded in six-well plates and with 80–90% confluence the cells were transfected with different recombinants described above, as well as vector control pIRES or pcDNA3.1 using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturers' instructions. Growth medium was replaced with medium containing 700 μg/ml G418 48 h later. About 12–14 days, three G418-resistant clones in each group were selected, clonally isolated and screened further for mRNA and protein expressions.

TOTAL RNA EXTRACTION AND QUANTITATIVE AND SEMIQUANTITATIVE PCR

RNA was isolated from the cells transfected with different recombinants using TRIzol reagent according to the manufacturers' instructions, then 2 μg RNA was reverse-transcribed in a 20 μl reaction with oligo dT primers using a reverse-transcription system (Promega). TaqMan probe and the primers were designed using the default settings of Primer Premier 5.0 (Premier Biosoft International). Each set of primers was used with a TaqMan probe labeled at the 5′ end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3′-end with the 6-carboxy-tetramethylrhodamine (TAMRA) quencher dye. The following primers and probes were employed for FPN1, HP or transferrin receptor 1 (TfR1), respectively. FPN1 forward 5′-TGGCCACTCTCTCTCCACTTG-3′, reverse 5′-GGACGTC-TGGGCCACTTTAAG-3′, probe 5′-ACGGAAACGGCCTCCTCTTGA-CAGC-3′; HP forward 5′-GCCTGACAATCCTGGGATATTTGA-3′, reverse 5′-TCTTGATGACTGGAACACTGAGAG-3′, probe 5′-CCAA-GCAGGCAGCCACAGAGAAGCA-3′; TfR1 forward 5′-GGGCACTA-GATTGGATACCTATGAG-3′, reverse 5′-GGTTCAATTCAACGTCA-TGGGTAAG-3′, probe 5′-CCACTTCCGCTGCTGTACGAACCAT-3′. Rat GAPDH gene was used as the reference: forward 5′-CCCCC-AATGTATCCGTTGTG-3′, reverse 5′-GTAGCCCAG GATGCCCTTTA-GT-3′, probe 5′-TCTGACATGCCGCCTGGAGAAACC-3′. Reactions were carried out on an ABI PRISM® 7500 Sequence Detection System using the relative quantification option of the SDS1.2.1 software (Applied Biosystems). Each reaction was run in triplicate with 2 μl (for FPN1) or 4 μl (for HP) sample in a total volume of 20 μl with primers and probes to a final concentration of 0.25 μmol/L. A

passive reference dye ROX II, which is not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions for normalization of the reporter signal. Amplication and detection were performed with the following conditions: an initial hold at 95°C for 10 s followed by 40 cycles at 95°C for 5 s and 60°C for 45 s.

To compare the changes of ferritin L, we used the following primers for PCR: forward 5′-CGCTTCCAGCCGCCTTTA-3′, reverse 5′TGGTTGCCCATCTTCTTG-3′. The amplified ferritin L fragment was 566 bp. Mouse β-actin was used as the reference: forward 5′-CTGTCCCTGTATGCCTCTG-3′, reverse 5′-TGATGTCACGCACGATTT-3′. The amplified β-actin fragment was 220 bp. Thermocycling was carried out as follows: 94°C for 5 min, then 32 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 45 s, followed by 72°C for 10 min.

WESTERN BLOTS

After three washes with cold PBS, cells were lysed directly on culture plates with lysis buffer containing 50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF and protease inhibitors (pepstatin 1 μg/ml, aprotinin 1 μg/ml, leupeptin 1 μg/ml). Eighty micrograms total proteins were separated using 8% SDS-polyacrylamide gels and then transferred by electroblotting into nitrocellulose membranes. After overnight blocking with 10% non-fat milk at 4°C, the membranes were incubated with rabbit-anti-rat FPN1 or HP antibody of 1:2,000 for 2 h at room temperature. Anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Cross-reactivity was visualized using ECL Western blots detection reagents and then analyzed through scanning densitometry by a Tanon Image System (Tanon, Shanghai, China). β-Actin was detected by rabbit-anti-β-actin monoclonal antibody (Sigma Chemical Co., 1:10,000) simultaneously with FPN1 or HP detection on the same blot. Among three clones with FPN1/HP overexpressions, two were chosen for the iron efflux assay in each group.

CALCEIN LOADING OF CELLS AND IRON EFFLUX ASSAY

Calcein-AM is a membrane-permeant, nonfluorescent molecule that becomes fluorescent upon intracellular cleavage by cytoplasmic esterases to calcein (membrane-impermeant). It is pH-independent, stable, and can be quenched rapidly by divalent metals and reversed easily by the chelators. It has a high affinity for both Fe^{2+} and Fe^{3+}, with affinity constants of 10^{-14} and 10^{-24} mol/L respectively, a good indicator of the so-called "labile iron pool" [Breuer et al., 1995]. When cells were exposed to DFO, a membrane-impermeant, strong and specific iron chelator, the intracellular iron were drained out to the medium, indicated by the increase in calcein fluorescence [Breuer et al., 1995; Tenopoulou et al., 2005]. Transfected cells were seeded to coverslips and after attachment they were incubated with calcein-AM (0.5 μmol/L final concentration) in HEPES-buffered saline (HBS, 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4) for 30 min at 37°C. The excess calcein on cell surface was washed out three times with HBS. CP, if there was any in the media supplement, was also fully washed away. The coverslips were mounted in a perfused heated chamber and cells were perfused with 100 μmol/L iron (ferrous sulfate in ascorbic acid solution) for 20 min. Calcein fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths and fluorescence intensity was measured every 3 min in the next 30 min while perfusing with 1 mmol/L DFO in HBS [Tenopoulou et al., 2005]. The mean fluorescence signal of 25–30 single cells in four separate fields was monitored at 200× magnification and processed with Fluoview5.0 Software. Among two clones with consistent increased iron efflux, one was eventually chosen for the following experiments in each group.

FLOW CYTOMETRIC MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) AND MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

To evaluate the intracellular ROS generation and mitochondrial transmembrane potential during the iron-induced oxidative stress, cells were incubated in HBS with $DCFH_2$-DA or rhodamine 123 (Rh123) and then analyzed by flow cytometry (Becton Dickinson, USA). The dye $DCFH_2$-DA can penetrate into cells and becomes hydrolyzed to non-fluorescent dichlorofluorescin (DCF). DCF then reacts with "ROS" to form the highly fluorescent dichlorofluorescein. The fluorescence intensity reflects the ROS generation inside cells [LeBel et al., 1992]. Rh123 assay is based on the selective accumulation of this dye in the mitochondria by facilitated diffusion. When the mitochondrial transmembrane potential is decreased, the amount of Rh123 that enters the mitochondria is decreased as there is no facilitated diffusion. Thus, the amount of Rh123 in the cells is an indicator of mitochondria function [Chen, 1988]. Transfected cells (1×10^5/ml) were seeded in six-well plates and after attachment they were then treated with vehicle or 1 mmol/L DFO for 3 h, followed by 100 μmol/L iron (ferrous sulfate) for 4 h at 37°C. After washing with HBS for three times, $DCFH_2$-DA (5 μmol/L) or Rh123 (5 μg/ml) was added and incubation was performed for 30 min at 37°C. The cells were re-suspended in 1 ml HBS. For analysis, excitation and emission wavelengths 488 and 525 nm, respectively were used to assess 10,000 cells from each sample. The intracellular ROS generation or the capacity of mitochondria to take up Rh123 was calculated as the difference in fluorescence intensity among cells transfected with different recombinants.

STATISTICAL ANALYSIS

Results are presented as means ± SEM. Differences between means in two groups were carried out by unpaired-samples *t*-test. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used to comparing difference between means in more than two groups. Efflux studies were carried out by the two ways ANOVA followed by Student–Newman–Keuls test and data were presented as mean ± SD. A probability value of $P < 0.05$ was considered to be statistically significant.

RESULTS

OVEREXPRESSION OF FPN1 AND HP DETERMINED BY REAL-TIME QUANTITATIVE PCR AND WESTERN BLOT

FPN1 and HP mRNA (Table I) and protein (Fig. 1) levels in MES23.5 cells transfected with pIRES, pIRES-FPN1 or pIRES-HP-FPN1 were analyzed by Taqman PCR and western blot. FPN1 mRNA expression was increased to 4.7-fold in pIRES-FPN1 group as compared to

TABLE I. Real-Time Quantitative PCR Analysis of FPN1 and HP mRNA Expression in Stably Transfected MES23.5 Cells

Group	FPN1	HP
pIRES-FPN1	$4.7 \pm 0.77^{*}$	1.01 ± 0.01
pIRES-HP-FPN1	$8.4 \pm 1.59^{*}$	$5.5 \pm 0.21^{*}$
pcDNA3.1-HP	1.0 ± 0.01	$5.6 \pm 0.11^{\#}$

Data were presented as mean ± SEM of six independent experiments indicating the fold changes of the mRNA expression in different groups.
$^{*}P < 0.01$, compared to pIRES group designated as 1.
$^{\#}P < 0.01$, compared to pcDNA3.1 group designated as 1.

vector control, and there were no changes observed for HP expression. There was a parallel 3.4-fold increase of FPN1 protein level. In pIRES-HP-FPN1 group, FPN1 and HP mRNA expression were increased to 8.4- and 5.5-fold, respectively, as compared to control, and protein levels increased to 2.9- (FPN1) and 3.2-fold (HP).

OVEREXPRESSION OF FPN1 AND/OR HP INCREASED IRON EFFLUX FROM MES23.5 CELLS

After calcein loading, there was a fast and steady intracellular fluorescence quenching when cells were perfused with 100 μmol/L iron, indicating the increased intracellular iron (data not shown). When the perfusion solution was changed to 1 mmol/L DFO, the quenching was readily reversed, with a significant higher increase in the fluorescence intensity in FPN1 or HP/FPN1 group as compared to vector control (Fig. 2). This indicated that overexpression of FPN1 alone or FPN1 and HP together can increase iron efflux. To better understand the role of HP in iron efflux, we created the stable cell line with HP overexpression by pcDNA3.1-HP transfection as described above. The result showed that there was a 5.62-fold increase of HP mRNA level (Table I) and a 3.8-fold increase of protein level (Fig. 3A). Cells with HP overexpression showed a significant increased iron efflux compared to pcDNA3.1 vector control (Fig. 3B).

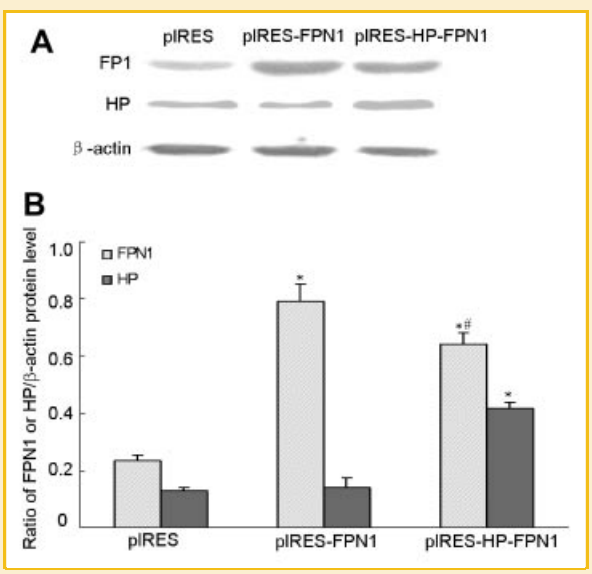

Fig. 1. FPN1 and HP protein expression in MES23.5 cells transfected with pIRES, pIRES-FPN1, or pIRES-HP-FPN1. A: FPN1 and HP expression was detected in vector control, pIRES-FPN1, and pIRES-HP/FPN1 transfected cells by western blot at 62 and 130 kDa, respectively, using antibodies against these proteins. B: Scanning densitometric quantification of protein expression. FPN1 expression was increased 3.4-fold in FPN1 group and 2.9-fold in HP/FPN1 group relative to vector control. There was no change in HP protein level in the FPN1 group and a 3.2-fold increase in the HP/FPN1 group relative to vector control. Data were presented as mean ± SEM of six independent experiments. $^{*}P < 0.01$, compared to pIRES group; $^{\#}P < 0.01$, compared to FPN1 group.

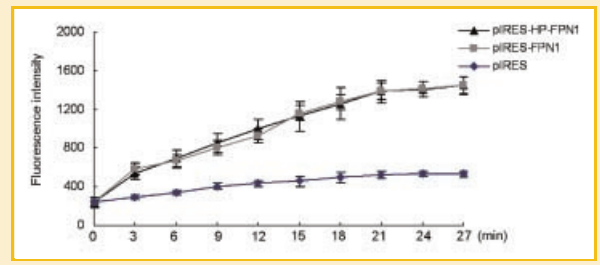

Fig. 2. Increased iron efflux from MES23.5 cells overexpressing FPN1 and HP/FPN1. Iron efflux was determined by the reverse quenching of calcein fluorescence, which is an indicator of intracellular iron level. The fluorescence intensity represented the mean value of 25–30 separate cells from four separate fields at each time point and was presented as mean ± SD of six independent experiments. When perfused with 1 mmol/L DFO following calcein loading, cells overexpressing FPN1 and HP/FPN1 exhibited a more rapid and steady increase in fluorescence than control, indicating increased iron efflux. $P < 0.001$, two-way ANOVA, $F = 34.284$, compared to vector control.

OVEREXPRESSION FPN1 AND/OR HP PREVENTED THE ELEVATION OF INTRACELLULAR IRON

Ferritin L is closely associated with cellular iron storage, and ferritin accumulation was observed under iron overloaded circumstances [Nemeth et al., 2004]. We examined its expression in iron overloaded MES23.5 cells to evaluate the intracellular iron level. As shown in Figure 4, there was a significant increase of ferritin L mRNA level with 1 mg/ml ferric ammonium citrate (FAC) incubation for 24 h in pIRES vector control group. FAC is a widely used iron overload reagent [Voloboueva et al., 2007; Messner et al., 2009]. We also observed the down-regulation of TfR1 mRNA, indicating that the intracellular iron overload resulted in increased iron storage as well as decreased iron uptake (Table II). However, there were no changes observed for ferritin L and TfR1 expression in FPN1 and HP/FPN1 groups with FAC treatment. Similarly, we observed the up-regulation of ferritin L and down-regulation of TfR1 mRNA levels with FAC treatment in pcDNA3.1 vector control group and no changes were observed in HP group (Fig. 4 and Table II). This result further suggested that high expression of FPN1 and/or HP could increase iron efflux and thus prevent elevation of intracellular iron under iron overloaded circumstances.

OVEREXPRESSION FPN1 AND/OR HP SUPPRESSED THE INCREASED ROS GENERATION INDUCED BY IRON

Our next question is whether over-expressed FPN1 and/or HP could suppress iron induced intracellular oxidative stress. Transfection of cells with different recombinants did not alter basal ROS production (data not shown). pIRES vector control group showed an increased

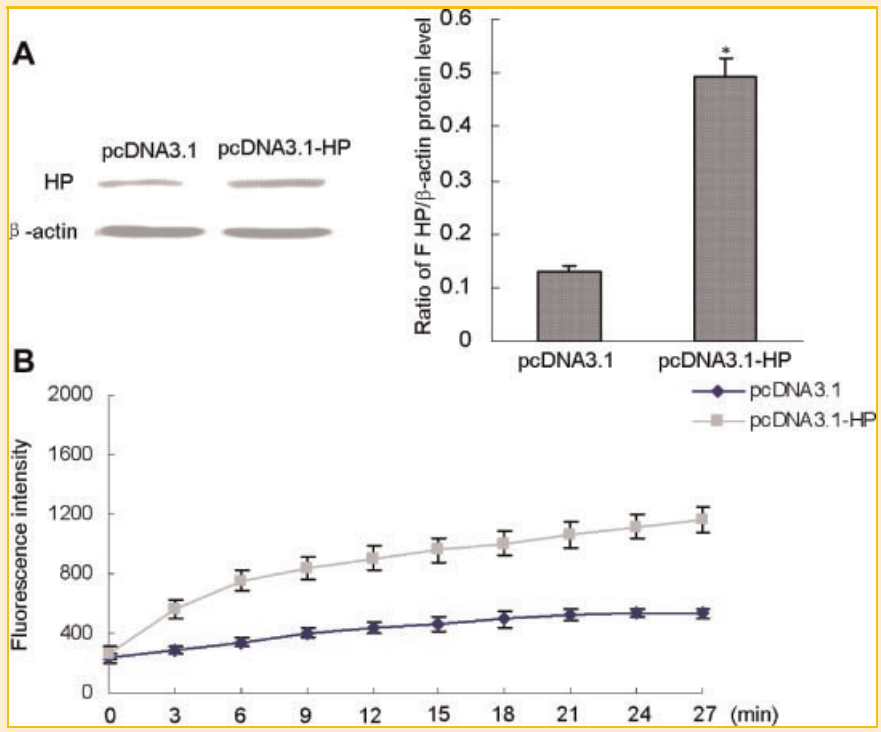

Fig. 3. Overexpression of HP in MES23.5 cells increases iron efflux. A: HP protein expression as determined by Western blot was significantly increased in pcDNA3.1-HP transfected cells as compared to vector control. Data are presented as mean ± SEM of six independent experiments. $^{*}P < 0.01$, compared to pcDNA3.1 group. B: Iron efflux was determined in cells with HP overexpression and vector control. There was a significant faster increase in fluorescence, indicating the increased iron efflux from cells overexpressing HP. $P < 0.001$, two-way ANOVA, $F = 61.266$, compared to vector control.

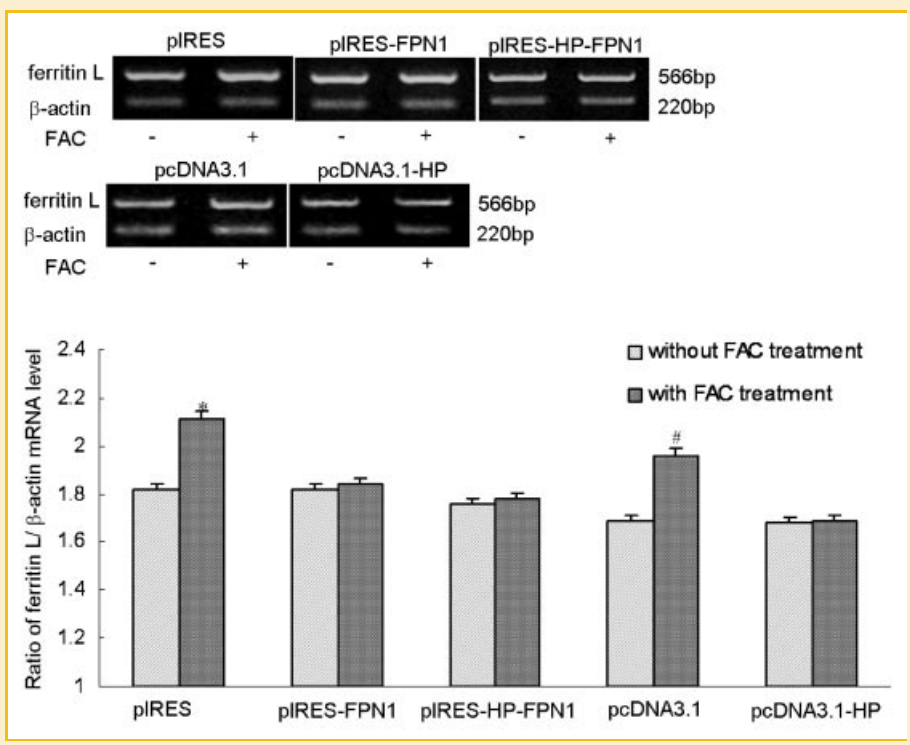

Fig. 4. Ferritin L mRNA expression in MES23.5 cells with FAC incubation for 24 h. After FAC incubation for 24 h, ferritin L mRNA levels were detected in cells transfected with different recombinants. In pIRES and pcDNA3.1 vector controls, ferritin L mRNA levels increased significantly after FAC incubation, while no changes were observed in FPN1, HP/FPN1, or HP groups. Data were presented as mean ± SEM of six independent experiments. $^{*}P < 0.01$, cells with FAC treatment versus without FAC treatment in pIRES group; $^{\#}P < 0.01$, cells with FAC treatment versus without FAC treatment in pcDNA3.1 group.

TABLE II. Real-Time Quantitative PCR Analysis of TfR1 mRNA Expression in Stably Transfected MES23.5 Cells With FAC Treatment

Group	TfR1
pIRES	$0.73 \pm 0.09^{*}$
pIRES-FPN1	1.12 ± 0.11
pIRES-HP-FPN1	1.11 ± 0.08
pcDNA3.1	$0.69 \pm 0.075^{\#}$
pcDNA3.1-HP	1.22 ± 0.23

Data were presented as mean ± SEM of six independent experiments indicating the fold changes of the mRNA expression with FAC treatment versus without FAC treatment in different groups.
$^{*}P < 0.01$, compared to pIRES group without FAC treatment designated as 1.
$^{\#}P < 0.01$, compared to pcDNA3.1 group without FAC treatment designated as 1.

ROS production about 64% over basal level when they were treated with 100 μmol/L iron for 4 h, which could be totally suppressed by 1 mmol/L DFO pretreatment. In FPN1, HP/FPN1 groups, ROS production was 35%, 29%, respectively. In pcDNA3.1 vector control group and HP group, ROS production was about 70% or 46% over basal level with iron treatment (Fig. 5). These results together with the above suggested that over-expressed FPN1 and/or HP could suppress iron induced ROS generation inside cells by decreasing intracellular iron.

OVEREXPRESSION FPN1 AND/OR HP RESTORED THE DECREASED MITOCHONDRIAL TRANSMEMBRANE POTENTIAL INDUCED BY IRON

Transfection of cells with different recombinants did not have any effect on mitochondrial transmembrane potential (data not shown). Most of pIRES transfected cells without iron treatment appear on the high Rh123 fluorescence field, indicative of intact, viable cells with high mitochondrial transmembrane potential. However, treatment with 100 μmol/L iron for 4 h induced a 19% reduction of Rh123-positive cells, indicated the damaged mitochondria. This effect could be fully abolished by DFO pretreatment. FPN1 or HP/FPN1 groups showed restored mitochondrial transmembrane potential by a less reduction of 11% or 8%, respectively (Fig. 6), indicating that increased iron efflux mediated by FPN1 and/or HP overexpression attenuated mitochondria dysfunction under iron over-loaded circumstances. We also observed that a significant reduction of 21% mitochondrial transmembrane potential in pcDNA3.1 group with iron treatment. However, in HP group, only a 13% reduction was observed.

DISCUSSION

Up to now, there has been little direct evidence for the mechanisms underlying iron efflux from neural cells in the CNS. Recent studies have demonstrated that FPN1 is widely expressed in

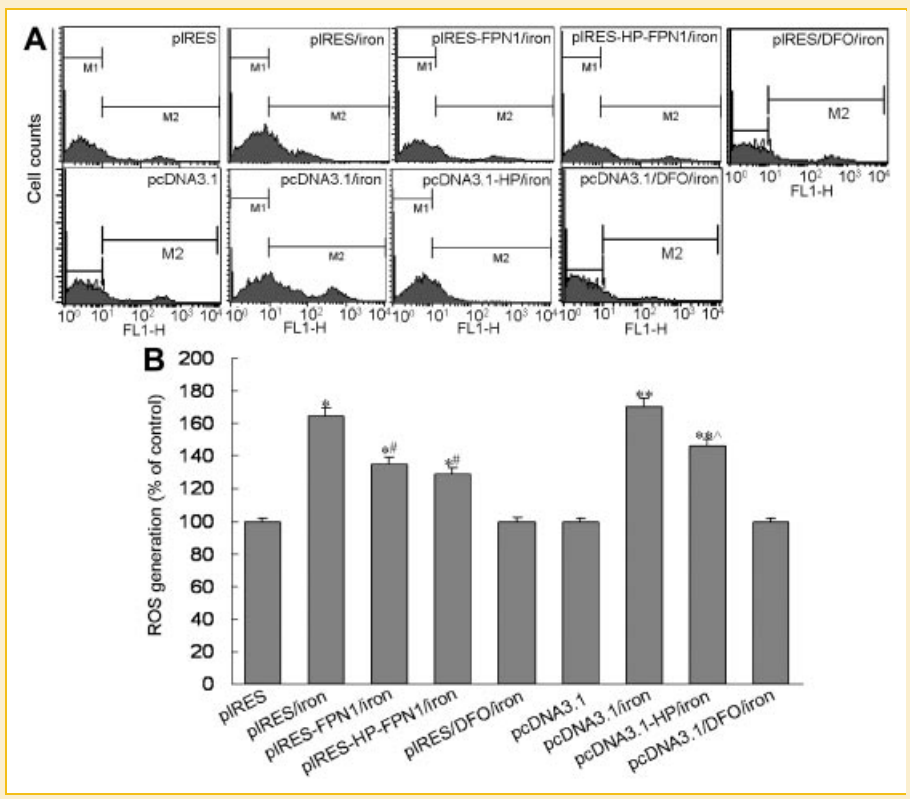

Fig. 5. ROS generation assessed by flow cytometry in MES23.5 cells with iron treatment for 4 h. A: Representatives of the fluorometric assay on ROS generation of different groups. In pIRES and pcDNA3.1 groups, 100 μmol/L iron treatment for 4 h induced a significant ROS generation compared to cells without iron treatment. However, ROS generation was suppressed significantly in FPN1, HP/FPN1, and HP groups treated with iron. Pretreatment with DFO could fully abolish the ROS generation induced by iron. B: Statistical analysis. Data were presented as mean ± SEM of six independent experiments. $^{*}P < 0.01$, $F = 30.274$, compared to cells without iron treatment in pIRES group; $^{\#}P < 0.01$, compared cells with iron treatment in pIRES group; $^{**}P < 0.01$, $F = 46.414$, compared to cells without iron treatment in pcDNA3.1 group; $^{\wedge}P < 0.01$, compared cells with iron treatment in pcDNA3.1 group.

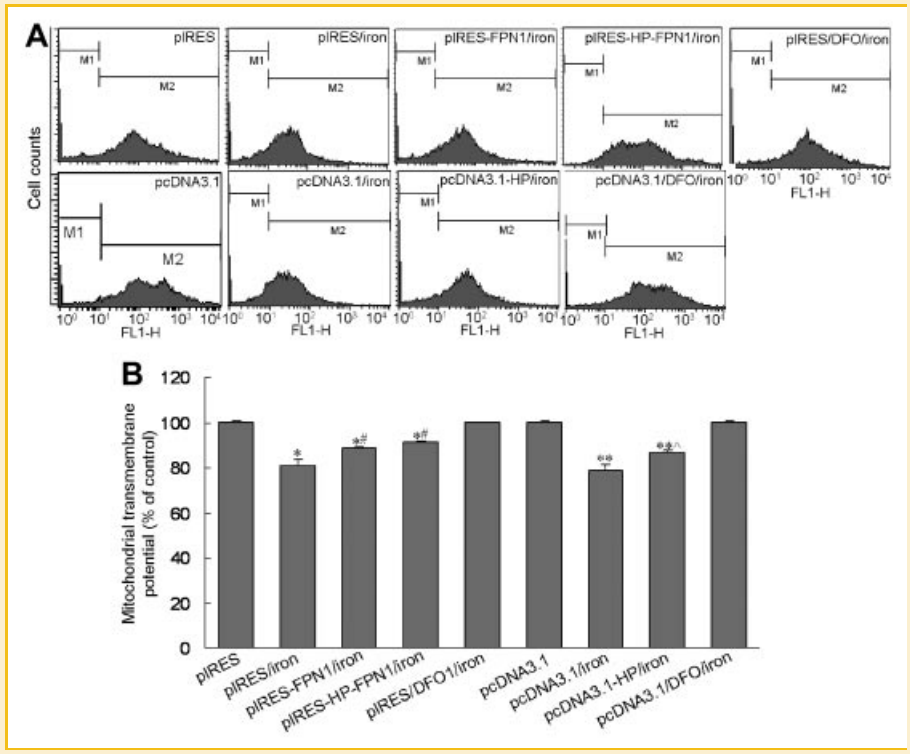

Fig. 6. Mitochondrial transmembrane potential ($\Delta\Psi$m) assessed by flow cytometry in MES23.5 cells with iron treatment for 4 h. A: Representatives of the fluorometric assay on $\Delta\Psi$m of different groups. In pIRES and pcDNA3.1 groups, 100 μmol/L iron treatment for 4 h caused a significant reduction of $\Delta\Psi$m. However, there was a significant recovery in FPN1, HP/FPN1, and HP group treated with iron. $\Delta\Psi$m changes induced by iron could be fully abolished by DFO pretreatment. B: Statistical analysis. Data were presented as mean ± SEM of six independent experiments. $^{*}P < 0.01$, $F = 15.571$, compared to cells without iron treatment in pIRES group; $^{\#}P < 0.01$, compared cells with iron treatment in pIRES group; $^{**}P < 0.01$, $F = 22.081$, compared to cells without iron treatment in pcDNA3.1 group; $^{\wedge}P < 0.01$, compared cells with iron treatment in pcDNA3.1 group.

brain [Burdo et al., 2001; Jiang et al., 2002; Wu et al., 2004; Moos et al., 2007], strongly supporting a role for this protein in that it may function to export iron in this tissue, playing a physiological role in brain iron homeostasis. The ferroxidase HP is a homologue of CP, facilitating iron export from cells by oxidizing ferrous to ferric iron, the only form that can be bound and carried by the blood transport protein transferrin. Soluble CP and glycosylphosphatidylinositol-anchored CP (GPI-CP) were shown to be essential for iron transport across the membrane of astrocytes from the brain in the presence of FPN1 [McKie et al., 2000; Jeong and David, 2003]. However, the lack of severe CNS iron overload in CP−/− mice, but the more severe phenotype observed in mice deficient in both CP and HP, indicate that its homologue-HP, plays a crucial role in CNS iron homeostasis [Hahn et al., 2004; Xu et al., 2004; Hadziahmetovic et al., 2008], despites of its relatively low expression in brain [Vulpe et al., 1999]. MES23.5 cell line, hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons, was often chosen as a dopaminergic neurons model in CNS [Colom et al., 1998; Xu et al., 2006; Cullen et al., 2009]. Both FPN1 and HP are co-localized in MES23.5 cells [Song et al., 2010], indicating that they might be involved in iron efflux process. We provide direct evidence that both FPN1 and HP could promote iron efflux process from MES23.5 dopaminergic cells in conditions of overexpression.

In the present study, increased iron export was observed in groups with overexpression of FPN1 alone, HP alone, and HP/FPN1, indicated by the fluorescent probe calcein. FPN1 is the sole cellular efflux channel for iron [Ganz, 2005]. The role of FPN1 in iron efflux from MES23.5 cells is further confirmed by the results that FPN1 knockdown significantly induced intracellular iron accumulation [Song et al., 2010]. It is interesting that we then observed HP itself could increase iron efflux. The fact that HP only has one transmembrane domain argues against its role as an iron transporter. We suppose that one possibility is that more HP oxidized the newly released ferrous iron and keep them in ferric forms, thus facilitated FPN1-mediated iron efflux. Another possibility might be that HP overexpression may increase iron export by stabilizing the membrane FPN1, as ferroxidase activity has been shown to be required for the stability of cell surface FPN1 expression [De Domenico et al., 2007]. We observed that cells in HP/FPN1 or FPN1 group exhibited similar increases in iron efflux relative to control, even though there was ~23% higher FPN1 protein expression in FPN1 group versus that in HP/FPN1 group. This might be due to the export threshold of the cells was already saturated at the FPN1 level observed in HP/FPN1 group and that the 23% increase in FPN1 expression observed in FPN group thus does not affect efflux any more. Alternatively, considering the export threshold of the cells was not saturated at the FPN1 level, the lower FPN1 protein levels in the HP/FPN1 group could be offset by the increased HP protein level. However, whether and how FPN1 and HP interact has yet to be determined [Anderson et al., 2002b]. In

addition, both endogenous and exogenous HP can co-localize with proteins of the endocytic apparatus and may thus exert ferroxidase function inside the cells [Li et al., 2003]. HP also may cycle, like transferrin receptor, between the cell surface and endocytic compartments [Roy and Enns, 2000]. Whether over-expressed cytoplasmic, cycling, or FPN1-interacting HP was involved in increased iron efflux is to be further elucidated.

The increased iron efflux mediated by FPN1 and HP overexpression indicated that these proteins might be able to down-regulate intracellular iron when highly expressed. Ferritin L is closely associated with cellular iron storage and it is induced by iron overload and thus plays a role in cellular labile iron balance [Cairo et al., 1985; Arnaud et al., 2006; Theil, 2006]. In our experiments we demonstrated that FPN1 and HP overexpression fully abolished the up-regulation of ferritin L after FAC incubation, consistent with other studies conducted in HEK293 cells [Nemeth et al., 2004]. Cells can handle excess iron by increasing iron storage by cytosolic ferritin, or by decreasing iron uptake by iron importer. TfR1, the main protein handling iron uptake, was post-transcriptionally regulated by iron through an iron responsive element/iron regulatory protein dependent mechanism. In our experiments, MES23.5 cells transfected with vector controls showed a significant decrease in TfR1 mRNA expression with FAC treatment. With such a regulating pathway, iron could not be imported from cells, thus prevent cellular iron overload. We observed that FPN1 and HP overexpression could fully prevent TfR1 down-regulation with iron overload. The lack of TfR1 down-regulation as well as ferritin L accumulation in cells overexpressing FPN1 and HP indicates that cytosolic iron was did not accumulate with FAC treatment because of the FPN1 and/or HP overexpression. This further supported the possibility that decreased FPN1 and HP expression might be involved in nigral iron accumulation in PD [Wang et al., 2007].

Iron plays a key role in the oxidative stress, as capable to catalyze H_2O_2 and form the highly reactive hydroxyl radicals resulting in oxidative damage [Jellinger et al., 1990]. Dopaminergic neurons are particularly vulnerable to mitochondrial dysfunction and oxidative stress, and dopamine itself can be an endogenous source of ROS because it produces H_2O_2 in the process of auto-oxidation [Cantuti-Castelvetri et al., 2003; Sulzer, 2007]. In the presence of excessive iron, further ROS formation damages the mitochondrial membrane, and such mitochondrial dysfunctions are implicated as key events in the pathogenic cascades leading to apoptosis or necrosis [Kroemer and Reed, 2000; Gong et al., 2004; Simpkins and Dykens, 2008]. As shown in our experiments, iron overload in MES23.5 dopaminergic cells caused significant mitochondria dysfunction and more ROS generation. FPN1 and HP overexpression lowered intracellular iron levels and thus attenuated iron-induced oxidative stress, indicated by the suppressed ROS generation and restored mitochondrial transmembrane potential. This is further confirmed by the present result that the DFO could fully abolished iron-induced oxidative damage, consistent with in vivo data that iron chelator could exert significant neuroprotective effects and prevent the progressive loss of dopaminergic neurons [Shachar et al., 2004; Youdim et al., 2004; Jiang et al., 2006], which may be a consequence of lowering increased intracellular iron.

In summary, in the present study we showed that FPN1 and/or HP promote iron efflux from a dopaminergic neuronal model in conditions of overexpression. Overexpression of FPN1 and/or HP could transport more iron outside, decrease intracellular iron levels, and thus protect cells from iron-induced oxidative stress. It has been demonstrated that iron chelation could be a valuable therapeutic approach in PD [Gaeta and Hider, 2005; Levenson et al., 2004], and our data further supported that agents with the ability to lower intracellular iron levels by increasing export could also be useful in iron-related neurodegenerative diseases.

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