



Pro-inflammatory cytokines modulate iron regulatory protein 1 expression and iron transportation through reactive oxygen/nitrogen species production in ventral mesencephalic neurons



Jia Wang¹, Ning Song¹, Hong Jiang, Jun Wang, Junxia Xie^{*}

Department of Physiology, Shandong Provincial Key Laboratory of Pathogenesis and Prevention of Neurological Disorders and State Key Disciplines: Physiology, Medical College of Qingdao University, Qingdao 266071, China

ARTICLE INFO

Article history:

Received 1 August 2012

Received in revised form 11 December 2012

Accepted 24 January 2013

Available online 31 January 2013

Keywords:

Interleukin-1 β

Tumor necrosis factor- α

Iron

Iron regulatory protein 1

Microglia

Neuron

ABSTRACT

Both inflammatory processes associated with microglia activation and abnormal iron deposit in dopaminergic neurons are involved in the pathogenesis of Parkinson's disease (PD). However, the relationship between neuroinflammation and iron accumulation was not fully elucidated. In the present study, we aimed to investigate whether the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) released by microglia, could affect cellular iron transportation in primary cultured ventral mesencephalic (VM) neurons. The results showed that IL-1 β or TNF- α treatment led to increased ferrous iron influx and decreased iron efflux in these cells, due to the upregulation of divalent metal transporter 1 with the iron response element (DMT1 + IRE) and downregulation of ferroportin1 (FPN1). Increased levels of iron regulatory protein 1 (IRP1), transferrin receptor 1 (TfR1) and hepcidin were also observed in IL-1 β or TNF- α treated VM neurons. IRP1 upregulation could be fully abolished by co-administration of radical scavenger *N*-acetyl-L-cysteine and inducible NO synthetase inhibitor *N*_ω-nitro-L-arginine methyl ester hydrochloride. Further experiments demonstrated that IL-1 β and TNF- α release was remarkably enhanced by iron load in activated microglia triggered by lipopolysaccharide or 1-methyl-4-phenylpyridinium (MPP⁺). In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice, salicylate application could not block DMT1 + IRE upregulation in dopaminergic neurons of substantia nigra. These results suggested that IL-1 β and TNF- α released by microglia, especially under the condition of iron load, might contribute to iron accumulation in VM neurons by upregulating IRP1 and hepcidin levels through reactive oxygen/nitrogen species production. This might provide a new insight into unraveling that microglia might aggravate this iron mediated neuropathologies in PD.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and consequently leading to dopamine (DA) depletion in the striatum. Increasing evidence suggests that neuro-inflammation, characterized by microglia activation and secretion of pro-inflammatory cytokines, is an active process in PD. Elevated cytokines interleukin 1 β (IL-1 β) and tumor necrosis factor- α (TNF- α)

were detected in the SN and striatum of PD patients and animal models [1,2]. Direct intra-parenchymal injection of TNF- α or IL-1 β induced dopaminergic neuron degeneration [3], and specific inhibition of IL-1 β or TNF- α attenuate the death of dopaminergic neurons in 6-hydroxydopamine (6-OHDA) or lipopolysaccharide (LPS) induced PD models [4,5]. The two pro-inflammatory cytokines may not only associate with PD pathogenesis by themselves, but actually aggravate PD involvement [6]. However, the mechanisms underlying elevated cytokines induced neurotoxicity are not fully elucidated.

Levels of total iron and ferric iron were first reported to respectively increase by 176% and 225% in the SNpc of PD patients relative to age-matched controls [7]. Compelling evidence exists that nigral abnormal iron metabolism, is one of the initial events that contribute to dopaminergic neuron degeneration in PD [8–10]. Especially in single dopaminergic neurons, iron levels are elevated in PD patients [11]. Identification of several iron transporters has expanded our knowledge of transmembrane iron traffic and disrupted intracellular iron homeostasis. Such proteins include divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1), which have tightly relationship with intracellular iron concentration. DMT1 was known to be responsible for

Abbreviations: DMT1, divalent metal transporter 1; FAC, ferric ammonium citrate; iNOS, inducible NO synthetase; IL-1 β , interleukin-1 β ; FPN1, ferroportin 1; IRP, iron regulatory protein; IRE, iron responsive element; LPS, lipopolysaccharide; MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; L-NAME, *N*_ω-nitro-L-arginine methyl ester hydrochloride; PD, Parkinson's disease; ROS, reactive oxygen species; SN, substantia nigra; TfR1, transferrin receptor 1; TNF- α , tumor necrosis factor- α ; TH, tyrosine hydroxylase; VM, ventral mesencephalon

^{*} Corresponding author. Tel.: +86 532 85955891; fax: +86 532 83780136.

E-mail address: jxixie@public.qd.sd.cn (J. Xie).

¹ These authors contributed equally to this work.

ferrous iron uptake and FPN1, known as MTP1, IREG 1, or SLC11A3, is a multiple transmembrane domain protein, which is the only cellular iron efflux channel described so far [12]. Our previous studies showed that upregulation of DMT1 with iron responsive element (IRE) and downregulation of FPN1, accounted for intracellular iron excess in 6-OHDA induced PD models [13–16].

PD patients showed amplified levels of pro-inflammatory cytokines in the SN as well as iron deposit. However, although effects of pro-inflammatory cytokines on iron transport was investigated in human monocytic cell lines [17,18], whether they were linked to dopaminergic neuron iron accumulation was not clarified. We supposed that mis-regulation of iron transporters and iron accumulation in dopaminergic neurons might be also attributable to the remarkably increased cytokines by microglia. Therefore, the present study was carried out to investigate the effects of pro-inflammatory cytokines on iron transportation and expressions of iron transporters in primary cultured ventral mesencephalic (VM) neurons, as well as the underlying mechanisms. To our knowledge, this is the first report demonstrating that pro-inflammation cytokines are associated with the disturbance of neuronal iron homeostasis.

2. Materials and methods

2.1. Materials

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guidelines for the Use of Animals in Neuroscience Research. Recombinant IL-1 β , TNF- α , LPS, Greiss reagent, ferric ammonium citrate (FAC), FeSO₄·7H₂O, *N*-acetyl-L-cysteine (NAC), *N*_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), 1-methyl-4-phenylpyridinium (MPP⁺), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and primary antibody against tyrosine hydroxylase (TH) were from Sigma Chemical Co. (St Louis, MO, USA). Primary antibodies against DMT1 + IRE, FPN1 and iron regulatory protein 1 (IRP1) were from ADI (San Antonio, TX, USA). Primary antibody against TfR1 was from Abcam (Cambridge, MA, USA). Carboxy-H₂DCFDA and calcein-AM were from Molecular Probes (Molecular Probes Inc., Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM)/F12 and B27 were from Gibco (Grand Island, NY, USA). All other chemicals and reagents were of the highest grade available from local commercial sources.

2.2. Primary cell cultures

2.2.1. VM neuron culture

Primary cultures of mesencephalic neurons were obtained from embryonic Sprague–Dawley rat mesencephalon as described previously [19,20], with some modifications. Briefly, regions of the ventral mesencephalon were dissected from embryonic 14-day rat brains and then mechanically dissociated with a pipette, until the tissue is dispersed. After centrifugation, cells were suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin and seeded at a density of 1×10^6 cells/mL on poly-D-lysine-coated coverslips or 12-well culture plates. Cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C for 18 h, and then the culture medium was changed to serum free DMEM/F12 supplemented with 2% B27. Cells were grown for a further 4 days before use. Neuron purity was about 95% based on immunofluorescence staining with a specific neuron marker microtubule-associated protein 2 (MAP2). Approximately 5% of the neurons exhibited TH positivity, which were dopaminergic neurons.

2.2.2. Microglia culture

Primary microglia were isolated from newborn Sprague–Dawley rat according to a previous study [21], with some modifications. Briefly, cerebral cortices were dissected from 1-day-old Sprague–Dawley rats,

stripped of the meninges and mechanically dissociated with a pipette, until it is dispersed. After centrifugation, cells were suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin and seeded in poly-D-lysine-coated 150 cm² flasks (5 brains/flask). Cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C. After 14 days, microglia were harvested in culture media containing serum by shaking the flasks at 180 rpm for 1 h. After centrifugation, the cell suspension was plated on 24-well plates at a density of 1×10^6 cells/mL for further experiments as described below. The purity of the microglial cultures was greater than 95% based on immunofluorescence staining with a specific microglial marker CD11b.

2.3. Calcein loading of cells and ferrous iron influx and efflux assay

Ferrous iron influx into neurons was determined by the quenching of calcein fluorescence as described before in our lab [13,22]. Neurons were seeded to coverslips and grown in serum-free medium with 10 ng/mL IL-1 β or TNF- α for 24 h. Then, they were loaded with calcein-AM (0.5 μ mol/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 the cell surface was washed out with HBS for 3 times, and the coverslips were mounted in a perfused chamber. Calcein fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths, and fluorescence intensity was measured every 3 min for 7 times while perfusing with 0.1 mmol/L ferrous iron (ferrous sulfate in ascorbic acid solution, 1:44 molar ratio, pH 6.0, prepared immediately prior to the experiments). Ascorbic acid maintained the reduced status of ferrous iron; in addition, ascorbate acted as a chelator to maintain the iron in solution. For iron efflux assay, neurons were perfused with 0.1 mmol/L ferrous iron for 20 min, then fluorescence intensity was measured every 3 min for 7 times while perfusing with 1 mmol/L deferoxamine (DFO), a membrane-impermeant, strong and specific iron chelator, and the intracellular iron was drained out to the medium, indicated by the increase in calcein fluorescence [23,24]. The mean fluorescence signal of 25–30 single cells in four separate fields was monitored at $\times 200$ magnification and processed with Fluoview 5.0 software.

2.4. Western blots

VM neurons were seeded in 12-well plates and incubated with IL-1 β or TNF- α as described above. After 3 washes with cold PBS, cells were lysed 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 phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (pepstatin 1 μ g/mL, aprotinin 1 μ g/mL, leupeptin 1 μ g/mL) for 30 min on ice and the insoluble material was removed by centrifugation (12,000 rpm, 20 min, 4 °C). The protein concentration was determined by the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). A total of sixty micrograms protein was separated using 10% SDS-polyacrylamide gels and then transferred onto PVDF membranes. After overnight blocking with 10% non-fat milk at 4 °C, the membranes were incubated with rabbit anti-rat DMT1 + IRE (1:500) or IRP1 (1:500) antibody for overnight at 4 °C, TfR1 (1:1000) or FPN1 antibody (1:8000) for 1 h at room temperature. Anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at 1:0,000 (Santa Cruz Biotechnology, Santa Cruz, CA). β -Actin was detected by mouse anti- β -actin monoclonal antibody (1:8000) according to a similar procedure to ensure equal samples of protein. Cross-reactivity was visualized using ECL Western blotting detection reagents and then analyzed through scanning densitometry by a UVP BioDoc-It Imaging System (UVP, Upland, USA).

2.5. Total RNA extraction and quantitative PCR

Total RNA was isolated by using Trizol Reagent (Invitrogen) from neurons treated as described above according to the manufacturer's

instructions. Then 2 µg total RNA was reversed transcribed in a 20 µL reaction with oligo-dT primers using reverse-transcription system (Promega). Quantitative RCR was employed to detect the changes of hepcidin. TaqMan probe and the primers were designed to sequences using the default settings of Primer Express 2.0 (PE Applied Biosystems). 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. Hepcidin forward 5'-CTC CGG CAA CAG ACG AGA C-3', reverse 5'-TTA CAG CAT TTA CAG CAG AAG AGG-3', probe 5'-TGG TGT CTC GCT TCC TTC GCT TCA G-3'. Rat GAPDH gene was used as the reference: forward 5'-CCC CCA ATG TAT CCG TTG TG-3', reverse 5'-GTA GCC CAG GAT GCC CTT TAG T-3', probe 5'-TCT GAC ATG CCG CCT GGA GAA ACC-3'. Reactions were carried out in an Eppendorf Realplex45 System using the relative quantification option of the Mastercycler ep Realplex 2.2 software. Each reaction was run in triplicate with 2 µL 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 was not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions for normalization of the reporter signals. Amplification 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.

2.6. Nitrite assay

Levels of the nitric oxide (NO) derivative nitrite were detected in the culture supernatants of VM neurons with the Griess reaction. After treatment with IL-1β or TNF-α as described above, supernatants were harvested and then mixed with equal volumes of 1 × Greiss Reagent (Sigma) in a 96-well plate. After incubation for 15 min at room temperature, the mixtures were read with a microplate reader at 540 nm and nitrite levels were extrapolated from a sodium nitrite standard curve.

2.7. Flow cytometric measurement of reactive oxygen species (ROS)

To evaluate the intracellular ROS generation during IL-1β or TNF-α induced oxidative stress, VM neurons were incubated in HBS with DCFH₂-DA and then analyzed by flow cytometry. The dye DCFH₂-DA can penetrate into cells and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCF). DCF then reacts with "ROS" to form the highly fluorescent dichlorofluorescein. The fluorescence intensity reflects the ROS generation inside cells [25]. After treatment with IL-1β or TNF-α as described above. DCFH₂-DA (5 µmol/L) was added and incubated for 30 min at 37 °C after washing with HBS for 3 times. And then neurons 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. Results were demonstrated as FL1-H (Fluorescence 1-Histogram), setting the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellquest Software.

2.8. Enzyme-linked immunosorbent assay (ELISA) of IL-1β and TNF-α

Microglia were cultured in 24-well plates, pre-incubated for 24 h with 100 µmol/L FAC, and then stimulated for another 24 h with 800 ng/mL LPS or 100 µmol/L MPP⁺ [26]. Culture supernatants were collected, and the concentrations of IL-1β and TNF-α were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) as described in the manufacturers' instructions.

2.9. Animal models and double-immunofluorescence labeling for DMT1 + IRE and TH

Eight to 10-week-old, male, C57BL/6 mice (Vital River Laboratory Animal Technology, Beijing, China) were housed with free access to

food and water. The mice received a single intraperitoneal injection of saline or salicylate (50 mg/kg) 5 min before a subcutaneous injection of MPTP (30 mg/kg, 4 times a day) or saline, and 2 days after MPTP or saline administration the mice were sacrificed. They were deeply anesthetized (8% chloral hydrate, i.p.) and transcardially perfused with normal saline and 4% paraformaldehyde in 0.1 mol/L PBS. Brain blocks containing the SN were sectioned coronally at 20 µm on a freezing microtome (Leica). Sections were rinsed in PBS first and then incubated in 10% goat serum overnight at 4 °C to block non-specific binding, followed by incubation overnight with primary antibodies consisting of DMT1 + IRE (1:100) along with TH (1:1000). The sections were then washed 3 times with PBS, and incubated in the second antibody consisting of Rhodamine-conjugated goat-anti-rabbit IgG (1:200) and FITC-conjugated goat-anti-mouse IgG (1:200) for 2 h at room temperature. After mounting on the coverslips, sections were examined using a Fluoview FV500 laser confocal scanning microscope (Olympus, Japan). Control sections incubated in a solution without primary antibodies showed no staining (data not shown).

2.10. Statistical analysis

Results are presented as means ± S.E.M. Differences between means in two groups were compared using the unpaired-samples *t* test. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used to compare difference between means in more than two groups. Influx and efflux studies were carried out using two-way ANOVA followed by the Student–Newman–Keuls test; data are presented as means ± S.D. A probability of *P* < 0.05 was taken to indicate statistical significance.

3. Results

3.1. IL-1β or TNF-α increased ferrous iron influx and decreased iron efflux of VM neurons

To elucidate whether IL-1β or TNF-α could affect intracellular iron homeostasis by modulating transmembrane transport, we used fluorescence dye calcein to measure the ferrous iron uptake and outflow process in VM neurons. After perfusing with 0.1 mmol/L ferrous iron, the fluorescence intensity significantly decreased in IL-1β or TNF-α treated neurons compared with the control (Fig. 1A), indicating that cells treated with IL-1β or TNF-α showed increased ferrous iron uptake. For efflux experiments, the fluorescence intensity reverse was analyzed when neurons were perfused with 1 mmol/L DFO. The quenching was more weakly reversed in IL-1β or TNF-α treated VM neurons compared with the control (Fig. 1B), indicating a decrease iron efflux process in these cells. The lower fluorescence intensity confirmed elevated iron levels in IL-1β or TNF-α treated VM neurons. There was no difference between IL-1β and TNF-α group in iron influx and efflux process. 10 ng/mL IL-1β or TNF-α had no effects on cell viability of VM neurons, indicating that the changes in iron influx/efflux are not due to a change in cell number.

3.2. IL-1β or TNF-α induced upregulation of IRP1 was responsible for upregulation of TfR1 and DMT1 + IRE, and downregulation of FPN1 in VM neurons

To determine the reason for the modulation of iron transportation in IL-1β or TNF-α treated neurons, we investigated the expression of DMT1 + IRE and FPN1 using Western blotting. DMT1 + IRE protein levels were significantly increased; while FPN1 levels were decreased compared with the control (Fig. 2).

Both DMT1 + IRE and FPN1 have IRE in their mRNAs, we hypothesized that IL-1β or TNF-α induced regulation of DMT1 + IRE and FPN1 might be dependent on IRE/IRP system. Therefore, we examined the expression of IRP1, an intracellular IRE-binding protein, in IL-1β or

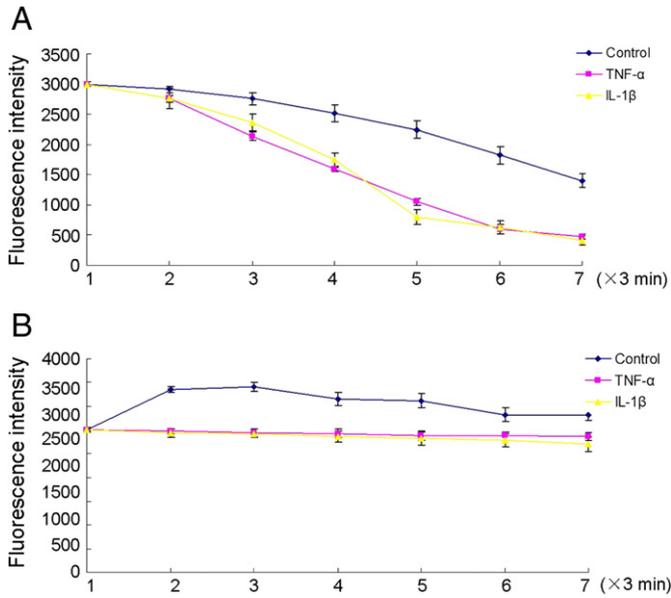


Fig. 1. IL-1β or TNF-α enhanced ferrous iron influx and attenuated iron efflux in VM neurons. Ferrous iron influx into VM neurons (A) was determined by the quenching and iron efflux (B) was determined by the reverse quenching of calcein fluorescence, which is an indicator of intracellular iron level. The fluorescence intensity represents the mean value of 25–30 cells from four separate fields at each time point and is presented as the mean ± SD of six independent experiments. (A) Neurons treated with 10 ng/mL IL-1β or TNF-α showed rapid and steady fluorescence quenching with ferrous iron perfusion. (B) The quenching was less reversed in IL-1β or TNF-α treated VM neurons with DFO perfusion (two-way ANOVA, $P < 0.01$, compared with the control).

TNF-α treated VM neurons. As expected, IRP1 protein level was increased 1.53 fold and 1.32 fold, respectively (Fig. 5). These results indicated that IRP1 upregulation might be responsible for IL-1β or TNF-α induced upregulation of DMT1 + IRE and downregulation of FPN1. Tfr1, another important IRP1 targeting protein in regulating intracellular iron homeostasis, was also upregulated in IL-1β or TNF-α treated VM neurons (Fig. 2).

Inflammation readily induced hepcidin expression by STAT3 signaling pathway mediated by pro-inflammatory cytokines [27]. As the receptor for hepcidin, FPN1 could be internalized and degraded [28]. We further analyzed hepcidin mRNA levels with real-time PCR. Hepcidin mRNA was found increased to 2.691 ± 0.657 or 3.320 ± 1.235 folds,

respectively, in VM neurons treated with IL-1β or TNF-α. The result indicated that in addition to IRP1/IRE system, hepcidin participated in the regulation of FPN1 expression in IL-1β and TNF-α treated VM neurons.

3.3. Oxidative stress and NO accounted for IL-1β or TNF-α induced IRP1 upregulation

Both oxidative stress and NO could activate IRP1 [29], therefore, we surmised that upregulation of IRP1 might be initiated by intracellular oxidative stress and/or NO induced by the pro-inflammatory cytokines. To test this hypothesis, we first quantified the changes of intracellular ROS levels and the concentration of nitrite in cells supernatants in IL-1β or TNF-α treated VM neurons. Increased levels of ROS and NO were observed, and pretreatment with radical scavenger NAC or inducible NO synthetase (iNOS) inhibitor L-NAME could fully abolish IL-1β or TNF-α induced ROS or nitrite augment (Figs. 3, 4). Co-administration of NAC and L-NAME could bring IRP1 expression returned to the basal level in IL-1β or TNF-α treated VM neurons, however, sole treatment with NAC or L-NAME just partially attenuated IL-1β or TNF-α induced IRP1 upregulation (Fig. 5). This indicated that both ROS and NO were responsible for IRP1 activation in these cells.

3.4. IL-1β and TNF-α levels were enhanced in activated microglia with iron load

The above results indicated IL-1β or TNF-α modulates iron homeostasis in VM neurons. We next quantified IL-1β and TNF-α in microglia culture supernatants to evaluate whether IL-1β and TNF-α released might be affected under the condition of iron load. Primary microglia were treated with 100 μmol/L FAC for 24 h, and then activated with LPS (800 ng/mL) or MPP⁺ (100 μmol/L) for another 48 h. The conditioned medium from the control or sole FAC treated microglia had barely detectable levels of any of the cytokines examined. Activated microglia by LPS showed a dramatic IL-1β and TNF-α secretion, however, MPP⁺ insult exhibited relatively weaker effects. In the presence of iron, these pro-inflammatory cytokine secretions induced by LPS or MPP⁺ were further enhanced (Fig. 6).

3.5. Salicylate application did not block DMT1 + IRE upregulation in dopaminergic neurons of the SN in MPTP-intoxicated mice

MPTP-intoxicated mice were used to implicate the role of microglia (or pro-inflammatory cytokines) in causing iron accumulation in

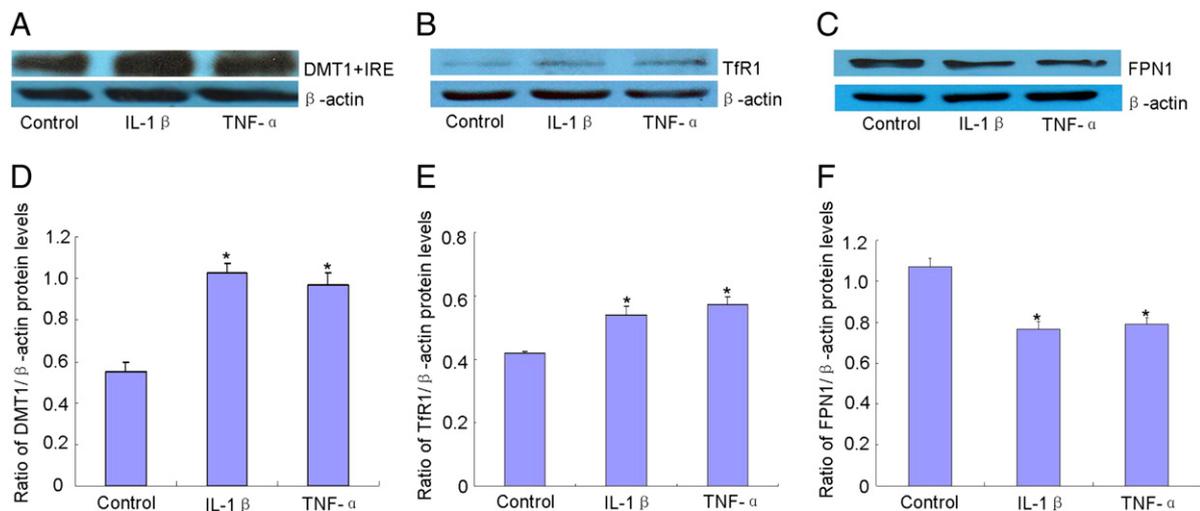


Fig. 2. IL-1β or TNF-α upregulated DMT1 + IRE and Tfr1 expressions and downregulated FPN1 expression in VM neurons. (A, B, C) DMT1 + IRE and Tfr1 were upregulated; while FPN1 was downregulated when VM neurons were treated with 10 ng/mL IL-1β or TNF-α for 24 h. β-actin was used as a loading control. (D, E, F) Statistical analysis. Data are presented as the ratio of DMT1 + IRE or FPN1 to β-actin. Each bar represented the mean ± S.E.M. of six independent experiments. * $P < 0.05$, compared with the control.

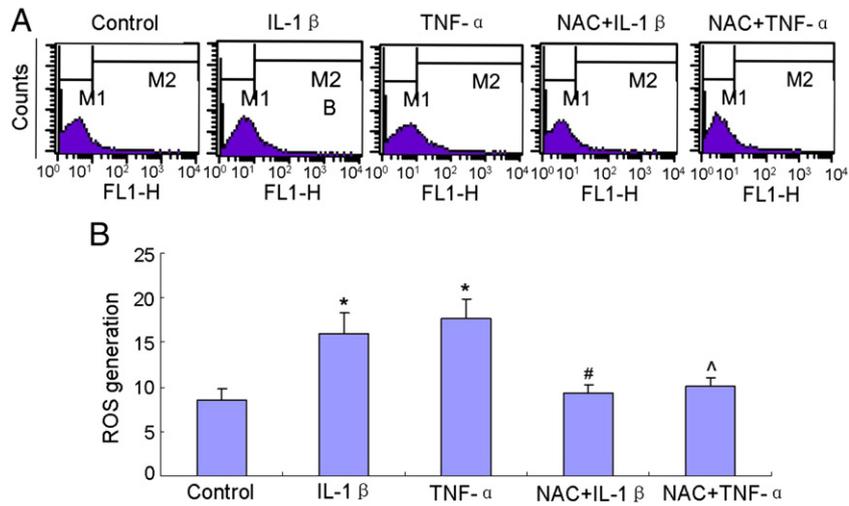


Fig. 3. IL-1 β or TNF- α increased ROS generation in VM neurons. (A) Representative fluorometric assays of ROS generation in different groups. VM neurons treated with 10 ng/mL IL-1 β or TNF- α for 24 h increased ROS generation compared with the control; pretreatment with NAC fully abolished these effects. (B) Statistical analysis. Data are presented as means \pm S.E.M. of six independent experiments. * P <0.01, compared with the control; # P <0.01, compared with IL-1 β treated group; ^ P <0.01, compared with TNF- α treated group.

neurons. As an anti-inflammatory agent which suppresses pro-inflammatory cytokines secretion [30–32], salicylate application could protect dopaminergic neurons against MPTP neurotoxicity [33,34]. The results showed that consistent in previous publication, 2 days after MPTP intoxication, DMT1 + IRE was upregulated in dopaminergic neurons of the SN [35]. However, 50 mg/kg salicylate application did not block this effect (Fig. 7).

4. Discussion

This study provides evidence that first, Tfr1 and DMT1 + IRE were upregulated and FPN1 was downregulated in IL-1 β or TNF- α treated VM neurons, which accounted for altered iron transportation and the elevation of iron levels. Second, IL-1 β or TNF- α aggravated hepcidin production, ROS and NO generation in VM neurons, the latter then resulted in activation of IRP1 and, led to mis-regulation of these iron transporters. Finally, cellular iron homeostasis influenced the secretion of IL-1 β and TNF- α in activated microglia.

Microglia respond to activating stimuli in the extracellular environment including endotoxin, chemokines, mis-folded proteins and serum factors by releasing substantial pro-inflammatory cytokines [36]. IL-1 β and TNF- α is major component of neuro-inflammation which plays a key role in PD. Increased levels of IL-1 β and TNF- α have been detected in the cerebrospinal fluid, as well as postmortem

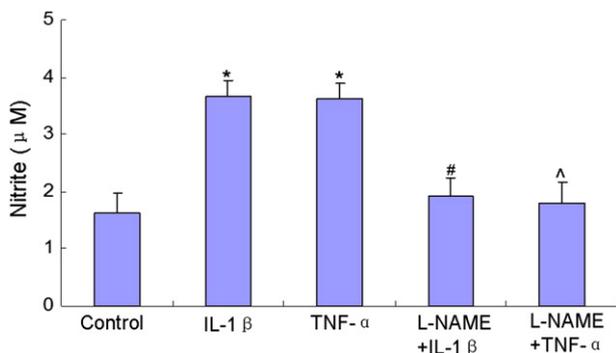


Fig. 4. IL-1 β or TNF- α increased nitrite generation in VM neurons conditioned medium. VM neurons treated with IL-1 β or TNF- α for 24 h increased nitrite generation compared with the control; pretreatment with L-NAME fully abolished these effects. Data are presented as means \pm S.E.M. of six independent experiments. * P <0.01, compared with control; # P <0.01, compared with IL-1 β treated group; ^ P <0.01, compared with TNF- α treated group.

brains (e.g. SN and striatum) of PD patients [1]. Elevation of IL-1 β and TNF- α was linked to dopaminergic neuron loss in both animal models and cultured cells [37]. However, currently, how IL-1 β and TNF- α

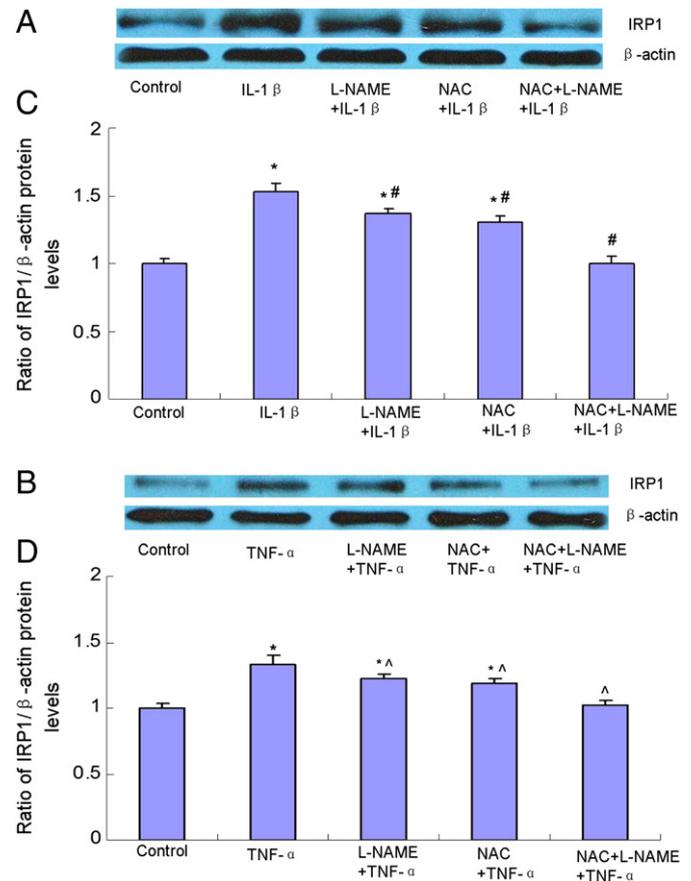


Fig. 5. Inhibition of ROS and NO blocked IRP1 activation in IL-1 β or TNF- α treated VM neurons. (A, B) The expression of IRP1 significantly increased in VM neurons treated with IL-1 β or TNF- α . These effects were partially attenuated by pretreatment with either NAC or L-NAME, however, fully abolished in cells pretreatment with both NAC and L-NAME. β -actin was used as a loading control. (C, D) Statistical analysis. Data are presented as the ratio of IRP1 to β -actin. Each bar represented the mean \pm S.E.M. of six independent experiments. * P <0.05, compared with the control, respectively; # P <0.05, compared with IL-1 β ; ^ P <0.05, compared with TNF- α .

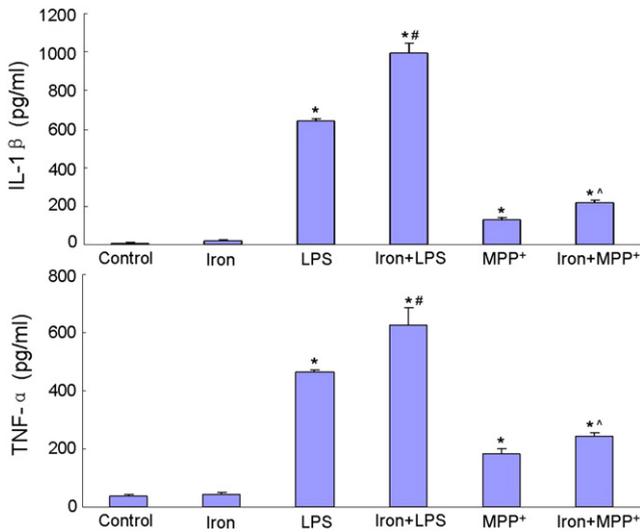


Fig. 6. FAC enhanced IL-1 β and TNF- α release from activated microglia triggered by LPS or MPP⁺. FAC alone did not increase the release of cytokines. Activation of microglia with LPS or MPP⁺ increased both IL-1 β and TNF- α release, and this was further enhanced by initial FAC incubation. * P <0.01, compared with the control; # P <0.05, compared with LPS; ^ P <0.05, compared with MPP⁺.

result in neurons' demise in vivo or in vitro is not fully elucidated. In our experiments, we found that IL-1 β or TNF- α increased ferrous iron influx and decreased iron efflux, thus leading to iron overload in VM

neurons. This might be linked to activated microglia and neuron interactions in PD pathogenesis, among which iron accumulation in neurons plays a key role [38]. Although suppression of pro-inflammatory cytokines secretion by salicylate did not block DMT1 upregulation in dopaminergic neurons of the SN in MPTP-intoxicated mice, together with the in vitro data that IL-1 β or TNF- α modulated iron transportation in neurons, we supposed that these pro-inflammatory cytokines might not be the main contributor, however, aggravate the nigral iron accumulation in PD in vivo.

Upregulation of DMT1 and downregulation of FPN1 were separately accounted for enhanced ferrous iron influx and attenuated iron efflux in neuronal cells [15,24]. In the present study, we observed that the expression of DMT1 increased and FPN1 decreased, and what's more, the level of IRP1 was elevated in IL-1 β or TNF- α treated neurons. IRPs could respond to cellular iron status and coordinate the iron homeostasis by binding to IRE. According to IRE-IRP theory, upregulated IRP1 binding to IRE increased DMT1 mRNA stability and repressed FPN1 mRNA translation. This is further confirmed by the result that another important IRP1 targeting protein with IRE, TfR1, was also upregulated in IL-1 β or TNF- α treated VM neurons. These results were consistent with our studies as well as other groups indicating that IRP1 regulates the expression levels of DMT1 and FPN1, and further confirmed by the fact and IRP1 knockdown induced a contrary regulation on these two transporters [13,39]. Furthermore, pro-inflammatory cytokines that induced hepcidin upregulation might have participated in the regulation of FPN1 expression in IL-1 β and TNF- α treated neurons.

IRPs could sense intracellular iron status and participate in the maintenance of cellular iron homeostasis. Much more abundant than IRP2 in most cells and tissues, usually IRP1 made a greater contribution

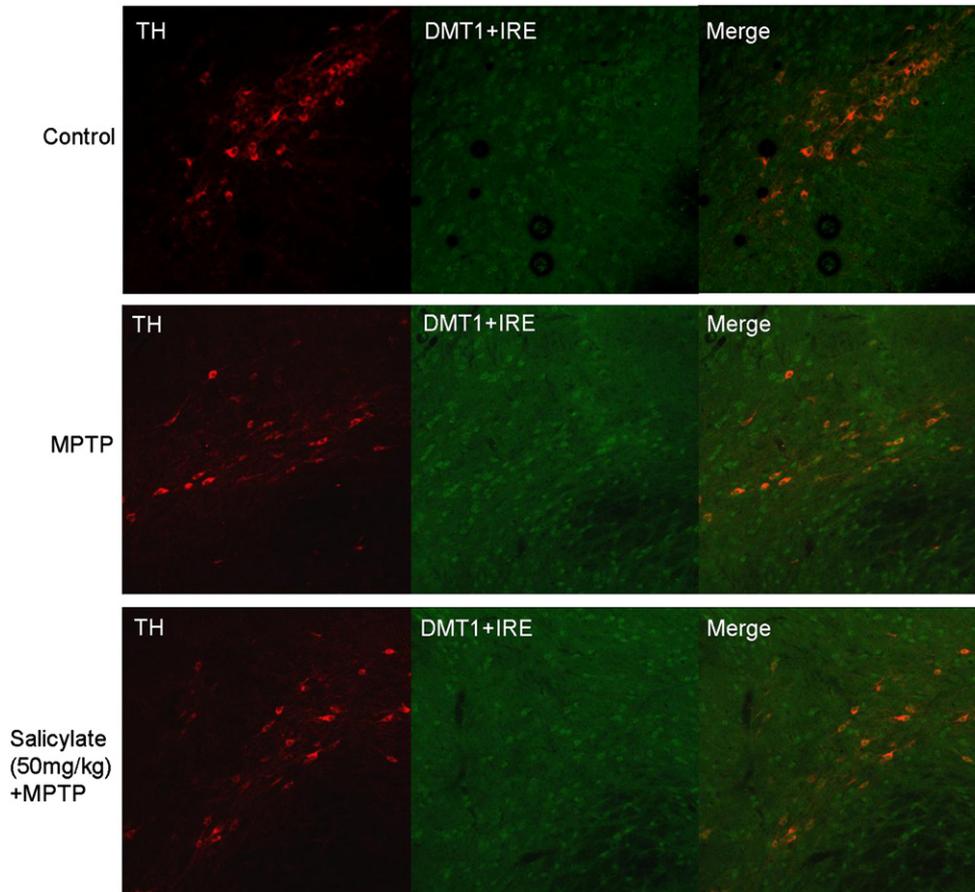


Fig. 7. DMT1 + IRE expression in the SN of MPTP-intoxicated mice. There was a modest expression of DMT1 + IRE (green) in TH (red)-positive dopaminergic neurons in control mice (top). DMT1 + IRE expression was upregulation in the SN 2 days after MPTP intoxication (middle), and salicylate application had no effects on DMT1 + IRE expression in TH-positive neurons (bottom).

to IRE-binding activity than IRP2, thus bond with higher affinity to the DMT1 + IRE [40,41]. Oxidative stress also preferentially influences IRP1 because of the presence of the Fe-S cluster in IRP1 [42]. Therefore, we mainly focused on IRP1 in our studies. IRP1 has two mutually exclusive activities, which either bind an IRE site or function as a cytosolic isoform of aconitase [43]. In iron-deficient cells, IRP1 binds with high affinity to IRE. Whereas in iron-replete cells, a 4Fe-4S cluster in IRP1 is fully assembled, and endowed with aconitase activity [44]. In addition to iron, reactive oxygen species and NO may activate IRP1 through disassembling the 4Fe-4S cluster, leading to the loss of cytoplasmic aconitase activity and the acquisition of high-affinity RNA binding capacity, thus confer IRP1's function as a posttranscriptional regulator [29]. As shown in our experiment, IL-1 β or TNF- α generated substantial NO and ROS, thus inducing activation of IRP1 in neurons. Both ROS and NO could enhanced hypoxia-inducible factor HIF-1 α activation [45–47], the latter then affects iron homeostasis by modulating several iron transporters, for example upregulating TfR1 [48]. However, recently it was reported that HIF could mediate IRP1 downregulation, as a dual-luciferase reporter assay provides direct evidence that HIF/HRE system was an essential link between IRP1 and HIF interactions [49]. We observed IRP1 upregulation in pro-inflammatory cytokines treated VM neurons, suggesting HIF might not be involved in this process. Actually, IRP1 acts as the dominant sensor and transducer of NO, as it stimulates the IRE-binding activity of IRP1 by targeting its Fe-S cluster [50]. IRP1 returned to basal level by pretreatment with iNOS inhibitor L-NAME and radical scavenger NAC together, however, either inhibitor alone just partially abolished IRP1 overexpression. Taken together, our results suggest that ROS and NO generated by IL-1 β or TNF- α induced activation of IRP1, leading to mis-regulation of iron transporters in VM neurons.

As demonstrated above, IL-1 β or TNF- α could modulate iron transport and resulted in iron accumulation in VM neurons. In vivo, iron load occurred in microglia in PD, which might be derived from damaged neurons. Endotoxin LPS was most commonly used to activate microglia both in vivo and in vitro [51]. By binding to toll-like receptor (TLR) 4, LPS initiates activation of NF- κ B that results in production of pro-inflammatory cytokines such as IL-1 β and TNF- α [52]. We supposed that iron could aggravate ROS generation, which promoted inflammatory signals leading to activation of NF- κ B and the consequent raising cytokine contents [53]. In the present study, we further revealed that the elevation of iron levels in activated microglia triggered by LPS or MPP⁺ induced more IL-1 β and TNF- α release. With this vicious cycle, activated microglia exacerbates neuronal iron accumulation and consequent degeneration in PD.

In summary, this study provides novel insight into unraveling that microglia might aggravate iron mediated neuropathologies by secreting IL-1 β and TNF- α in PD. Oxidative stress and NO induced by IL-1 β or TNF- α activated IRP1, regulated expression of TfR1, DMT1 + IRE and FPN1, thus leading to neuronal iron accumulation and even demise. This process was enhanced when microglia were iron-loaded. We supposed this might be extremely deleterious when microglia activation and iron deposit simultaneously occurred in PD. Epidemiological studies reported that chronic administration of aspirin or other NSAIDs decrease the risk of developing PD [54]. Our experiments provide powerful evidence that anti-inflammation and iron chelation could protect dopaminergic neurons by maintaining neuronal iron homeostasis, and show lights on the co-administration of anti-inflammatory agents and iron chelators could be a valuable therapeutic approach in PD.

Acknowledgements

This work was supported by grants from the National Program of Basic Research sponsored by the Ministry of Science and Technology of China (2011CB504102), the National Natural Science Foundation of China (30930036, 31171031, 30900477) and the Department of Science and Technology of Shandong Province (ZR2012HZ005, BS2010SW005).

References

- T. Nagatsu, M. Mogi, H. Ichinose, A. Togari, Changes in cytokines and neurotrophins in Parkinson's disease, *J. Neural Transm. Suppl.* (2000) 277–290.
- A.L. Bartels, K.L. Leenders, Neuroinflammation in the pathophysiology of Parkinson's disease: evidence from animal models to human in vivo studies with [¹¹C]-PK11195 PET, *Mov. Disord.* 22 (2007) 1852–1856.
- P.M. Carvey, E.Y. Chen, J.W. Lipton, C.W. Tong, Q.A. Chang, Z.D. Ling, Intra-parenchymal injection of tumor necrosis factor- α and interleukin 1- β produces dopamine neuron loss in the rat, *J. Neural Transm.* 112 (2005) 601–612.
- M.K. McCoy, T.N. Martinez, K.A. Ruhn, D.E. Szymkowski, C.G. Smith, B.R. Botterman, K.E. Tansey, M.G. Tansey, Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease, *J. Neurosci.* 26 (2006) 9365–9375.
- M.C. Pott Godoy, R. Tarelli, C.C. Ferrari, M.I. Sarchi, F.J. Pitossi, Central and systemic IL-1 exacerbates neurodegeneration and motor symptoms in a model of Parkinson's disease, *Brain* 131 (2008) 1880–1894.
- M. Menza, R.D. Dobkin, H. Marin, M.H. Mark, M. Gara, K. Bienfait, A. Dicke, A. Kusnekov, The role of inflammatory cytokines in cognition and other non-motor symptoms of Parkinson's disease, *Psychosomatics* 51 (2010) 474–479.
- E. Sofic, P. Riederer, H. Heinsen, H. Beckmann, G.P. Reynolds, G. Hebenstreit, M.B. Youdim, Increased iron (III) and total iron content in post mortem substantia nigra of parkinsonian brain, *J. Neural Transm.* 74 (1988) 199–205.
- Y. Ke, Z.M. Qian, Brain iron metabolism: neurobiology and neurochemistry, *Prog. Neurobiol.* 83 (2007) 149–173.
- D.W. Lee, J.K. Andersen, Iron elevations in the aging Parkinsonian brain: a consequence of impaired iron homeostasis? *J. Neurochem.* 112 (2010) 332–339.
- J. Sian-Hulsmann, S. Mandel, M.B. Youdim, P. Riederer, The relevance of iron in the pathogenesis of Parkinson's disease, *J. Neurochem.* 118 (2011) 939–957.
- A.E. Oakley, J.F. Collingwood, J. Dobson, G. Love, H.R. Perrott, J.A. Edwardson, M. Elstner, C.M. Morris, Individual dopaminergic neurons show raised iron levels in Parkinson disease, *Neurology* 68 (2007) 1820–1825.
- T. Ganz, Cellular iron: ferroportin is the only way out, *Cell Metab.* 1 (2005) 155–157.
- H. Jiang, N. Song, H. Xu, S. Zhang, J. Wang, J. Xie, Up-regulation of divalent metal transporter 1 in 6-hydroxydopamine intoxication is IRE/IRP dependent, *Cell Res.* 20 (2010) 345–356.
- J. Wang, H. Jiang, J.X. Xie, Ferroportin1 and hephaestin are involved in the nigral iron accumulation of 6-OHDA-lesioned rats, *Eur. J. Neurosci.* 25 (2007) 2766–2772.
- N. Song, H. Jiang, J. Wang, J.X. Xie, Divalent metal transporter 1 up-regulation is involved in the 6-hydroxydopamine-induced ferrous iron influx, *J. Neurosci. Res.* 85 (2007) 3118–3126.
- N. Song, J. Wang, H. Jiang, J. Xie, Ferroportin1 and hephaestin overexpression attenuate iron-induced oxidative stress in MES23.5 dopaminergic cells, *J. Cell. Biochem.* 110 (2010) 1063–1072.
- M. Fahmy, S.P. Young, Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA, *Biochem. J.* 296 (Pt 1) (1993) 175–181.
- S. Ludwiczek, E. Aigner, I. Theurl, G. Weiss, Cytokine-mediated regulation of iron transport in human monocytic cells, *Blood* 101 (2003) 4148–4154.
- K.S. McNaught, C. Mytilineou, R. Jnabaptiste, J. Yabut, P. Shashidharan, P. Jennert, C.W. Olanow, Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures, *J. Neurochem.* 81 (2002) 301–306.
- X. Liu, X.L. Fan, Y. Zhao, G.R. Luo, X.P. Li, R. Li, W.D. Le, Estrogen provides neuroprotection against activated microglia-induced dopaminergic neuronal injury through both estrogen receptor- α and estrogen receptor- β in microglia, *J. Neurosci. Res.* 81 (2005) 653–665.
- R.S. Roque, R.B. Caldwell, Isolation and culture of retinal microglia, *Curr. Eye Res.* 12 (1993) 285–290.
- S. Zhang, J. Wang, N. Song, J. Xie, H. Jiang, Up-regulation of divalent metal transporter 1 is involved in 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis in MES23.5 cells, *Neurobiol. Aging* 30 (2009) 1466–1476.
- M. Tenopoulou, P.T. Doulias, A. Barbouti, U. Brunk, D. Galaris, Role of compartmentalized redox-active iron in hydrogen peroxide-induced DNA damage and apoptosis, *Biochem. J.* 387 (2005) 703–710.
- N. Song, J. Wang, H. Jiang, J. Xie, Ferroportin 1 but not hephaestin contributes to iron accumulation in a cell model of Parkinson's disease, *Free Radic. Biol. Med.* 48 (2010) 332–341.
- C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress, *Chem. Res. Toxicol.* 5 (1992) 227–231.
- X. Zhang, N. Surguladze, B. Slagle-Webb, A. Cozzi, J.R. Connor, Cellular iron status influences the functional relationship between microglia and oligodendrocytes, *Glia* 54 (2006) 795–804.
- A. Pietrangolo, Hepcidin in human iron disorders: therapeutic implications, *J. Hepatol.* 54 (2011) 173–181.
- E. Nemeth, M.S. Tuttle, J. Powelson, M.B. Vaughn, A. Donovan, D.M. Ward, T. Ganz, J. Kaplan, Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization, *Science* 306 (2004) 2090–2093.
- G. Rathnasamy, E.A. Ling, C. Kaur, Iron and iron regulatory proteins in amoeboid microglial cells are linked to oligodendrocyte death in hypoxic neonatal rat periventricular white matter through production of proinflammatory cytokines and reactive oxygen/nitrogen species, *J. Neurosci.* 31 (2011) 17982–17995.
- H. Hackstein, A.E. Morelli, A.T. Larregina, R.W. Ganster, G.D. Papworth, A.J. Logar, S.C. Watkins, L.D. Faló, A.W. Thomson, Aspirin inhibits in vitro maturation and in

- vivo immunostimulatory function of murine myeloid dendritic cells, *J. Immunol.* 166 (2001) 7053–7062.
- [31] R.E. Shackelford, P.B. Alford, Y. Xue, S.F. Thai, D.O. Adams, S. Pizzo, Aspirin inhibits tumor necrosis factor- α gene expression in murine tissue macrophages, *Mol. Pharmacol.* 52 (1997) 421–429.
- [32] I. Tegeeder, J. Pfeilschifter, G. Geisslinger, Cyclooxygenase-independent actions of cyclooxygenase inhibitors, *FASEB J.* 15 (2001) 2057–2072.
- [33] B. Ferger, P. Teismann, C.D. Earl, K. Kuschinsky, W.H. Oertel, Salicylate protects against MPTP-induced impairments in dopaminergic neurotransmission at the striatal and nigral level in mice, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 360 (1999) 256–261.
- [34] K.P. Mohanakumar, D. Muralikrishnan, B. Thomas, Neuroprotection by sodium salicylate against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity, *Brain Res.* 864 (2000) 281–290.
- [35] J. Salazar, N. Mena, S. Hunot, A. Prigent, D. Alvarez-Fischer, M. Arredondo, C. Duyckaerts, V. Sazdovitch, L. Zhao, L.M. Garrick, M.T. Nunez, M.D. Garrick, R. Raisman-Vozari, E.C. Hirsch, Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 18578–18583.
- [36] C.M. Long-Smith, A.M. Sullivan, Y.M. Nolan, The influence of microglia on the pathogenesis of Parkinson's disease, *Prog. Neurobiol.* 89 (2009) 277–287.
- [37] D.A. Gayle, Z. Ling, C. Tong, T. Landers, J.W. Lipton, P.M. Carvey, Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor- α , interleukin-1 β , and nitric oxide, *Brain Res. Dev. Brain Res.* 133 (2002) 27–35.
- [38] S. Lehnardt, Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury, *Glia* 58 (2010) 253–263.
- [39] J. Kato, M. Kobune, S. Ohkubo, K. Fujikawa, M. Tanaka, R. Takimoto, K. Takada, D. Takahari, Y. Kawano, Y. Kohgo, Y. Niitsu, Iron/IRP-1-dependent regulation of mRNA expression for transferrin receptor, DMT1 and ferritin during human erythroid differentiation, *Exp. Hematol.* 35 (2007) 879–887.
- [40] T. Christova, D.M. Templeton, Effect of hypoxia on the binding and subcellular distribution of iron regulatory proteins, *Mol. Cell. Biochem.* 301 (2007) 21–32.
- [41] H. Gunshin, C.R. Allerson, M. Polycarpou-Schwarz, A. Rofts, J.T. Rogers, F. Kishi, M.W. Hentze, T.A. Rouault, N.C. Andrews, M.A. Hediger, Iron-dependent regulation of the divalent metal ion transporter, *FEBS Lett.* 509 (2001) 309–316.
- [42] R.S. Eisenstein, Iron regulatory proteins and the molecular control of mammalian iron metabolism, *Annu. Rev. Nutr.* 20 (2000) 627–662.
- [43] K. Pantopoulos, G. Weiss, M.W. Hentze, Nitric oxide and oxidative stress (H_2O_2) control mammalian iron metabolism by different pathways, *Mol. Cell. Biol.* 16 (1996) 3781–3788.
- [44] D.J. Haile, T.A. Rouault, J.B. Harford, M.C. Kennedy, G.A. Blondin, H. Beinert, R.D. Klausner, Cellular regulation of the iron-responsive element binding protein: disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding, *Proc. Natl. Acad. Sci. U. S. A.* 89 (1992) 11735–11739.
- [45] B. Brix, J.R. Mesters, L. Pellerin, O. Jöhren, Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1 α -mediated target gene activation, *J. Neurosci.* 32 (2012) 9727–9735.
- [46] R. Chowdhury, L.C. Godoy, A. Thiantanawat, L.J. Trudel, W.M. Deen, G.N. Wogan, Nitric oxide produced endogenously is responsible for hypoxia-induced HIF-1 α stabilization in colon carcinoma cells, *Chem. Res. Toxicol.* 25 (2012) 2194–2202.
- [47] H. Niecknig, S. Tug, B.D. Reyes, M. Kirsch, J. Fandrey, U. Berchner-Pfannschmidt, Role of reactive oxygen species in the regulation of HIF-1 by prolyl hydroxylase 2 under mild hypoxia, *Free Radic. Res.* 46 (2012) 705–717.
- [48] L. Tacchini, E. Gammella, C. De Ponti, S. Recalcati, G. Cairo, Role of HIF-1 and NF- κ B transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals, *J. Biol. Chem.* 283 (2008) 20674–20686.
- [49] Q.Q. Luo, D. Wang, M.Y. Yu, L. Zhu, Effect of hypoxia on the expression of iron regulatory proteins 1 and the mechanisms involved, *IUBMB Life* 63 (2011) 120–128.
- [50] A. Stys, B. Galy, R.R. Starzynski, E. Smuda, J.C. Drapier, P. Lipinski, C. Bouton, Iron regulatory protein 1 outcompetes iron regulatory protein 2 in regulating cellular iron homeostasis in response to nitric oxide, *J. Biol. Chem.* 286 (2011) 22846–22854.
- [51] D.M. Bronstein, I. Perez-Otano, V. Sun, S.B. Mullis Sawin, J. Chan, G.C. Wu, P.M. Hudson, L.Y. Kong, J.S. Hong, M.K. McMillian, Glia-dependent neurotoxicity and neuroprotection in mesencephalic cultures, *Brain Res.* 704 (1995) 112–116.
- [52] M.A. Dobrovolskaia, S.N. Vogel, Toll receptors, CD14, and macrophage activation and deactivation by LPS, *Microbes Infect.* 4 (2002) 903–914.
- [53] R.R. Crichton, S. Wilmet, R. Legssyer, R.J. Ward, Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells, *J. Inorg. Biochem.* 91 (2002) 9–18.
- [54] H. Chen, E. Jacobs, M.A. Schwarzschild, M.L. McCullough, E.E. Calle, M.J. Thun, A. Ascherio, Nonsteroidal antiinflammatory drug use and the risk for Parkinson's disease, *Ann. Neurol.* 58 (2005) 963–967.