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Increased Divalent Metal Transporter 1 Expression Might Be Associated with the Neurotoxicity of L-DOPA

Yan-Zhong Chang, Ya Ke, Jun-Rong Du, Georges M. Halpern, Kwok-Ping Ho, Li Zhu, Xiao-Song Gu, You-Jia Xu, Qin Wang, Lian-Zhi Li, Chen-Yuen Wang, and Zhong-Ming Qian

Department of Applied Biology and Chemical Technology, and National Key Laboratory of Chinese Medicine and Molecular Pharmacology (Shenzhen), Hong Kong Polytechnic University, Kowloon, Hong Kong (Y.-Z.C., Y.K., J.-R.D., G.M.H., K.-P.H., L.Z., Y.-J.X., Q.W., L.-Z.L., C.-Y.W., Z.-M.Q.); the Biotechnology Laboratory, Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada (Y.K.); and the Key Laboratory of Nerve Regeneration, Nanton University, Nanton, Jiangsu, People's Republic of China (L.Z., X.-S.G., Z.-M.Q.)

ABSTRACT

Based on the available data, we speculated that changes in brain iron metabolism induced by L-DOPA might be associated with the neurotoxicity of L-DOPA. To investigate this possibility, the effects of L-DOPA on the expression of iron influx proteins [transferrin receptor (TfR) and divalent metal transporter 1 (DMT1)], iron efflux protein (ferroportin 1), and iron uptake in C6 glioma cells were determined in this study using Northern blot and Western blot analysis and the calcein method. The findings showed that treatment of C6 cells with different concentrations of L-DOPA (0–100 μM) did not affect the expression of mRNA and protein of TfR and DMT1 with iron-responsive element (+IRE) and protein of ferroportin 1. However, a significant increase in the expression of DMT1(−IRE) mRNA and protein was found in cells treated, respectively, with 10 and 30 μM L-DOPA (mRNA) and 1, 5, 10 and 30 μM L-DOPA (protein). The increase in DMT(−IRE) protein induced by L-DOPA treatment was in parallel with the increase in DMT(−IRE) mRNA. The levels of DMT1(−IRE) mRNA and protein peaked in the cells treated with 10 μM L-DOPA and then decreased progressively with increasing concentrations of L-DOPA. Further study demonstrated that treatment of the cells with 10 μM L-DOPA induced a significant increase in ferrous uptake by C6 glioma cells. The findings suggested that the increased DMT1(−IRE) expression might be partly associated with the neurotoxicity of L-DOPA. Clinical relevance of the findings needs to be investigated further.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized mainly by degeneration of dopamine-containing neurons. The affected sections of the brain are therefore deprived of adequate amounts of the neurotransmitter dopamine (Foley, 2000). Because dopamine itself cannot access the brain directly, its natural precursor, L-DOPA, is used in clinical treatment of patients with PD. L-DOPA remains the most effective treatment for the symptomatic control of PD (Dunnett and Björklund, 1999; LeWitt and Nyholm, 2004). However, it was reported that long-term administration of high oral doses could cause drug-induced involuntary movements, on-off fluctuation of efficacy, and dyskinesias (Jenner and Brin, 1998; Foster and Hoffer, 2004).

The causes of the long-term side effects caused by L-DOPA treatment of PD are not yet completely known. Based on available data, we speculated that changes in brain iron metabolism induced by L-DOPA might be associated with neurotoxicity or side effects of L-DOPA. First, it has been suggested that changes in trace metal concentrations in the brain may be related to the long-term toxicity of L-DOPA (Weiner et al., 1978). Second, a clinical study (Boll et al., 1999) demonstrated that L-DOPA can significantly affect brain ceruloplasmin, a major factor in the regulation of regional brain iron content and that cerebrospinal fluid ferroxidase (CP) in L-DOPA-treated patients with PD was significantly higher than that in patients with PD who were not

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ABBREVIATIONS: PD, Parkinson's disease; DMT1, divalent metal transporter 1; IRE, iron-responsive element; DMT1(+IRE), divalent metal transporter 1 with iron-responsive element; DMT1(−IRE), divalent metal transporter 1 without iron-responsive element; BP bathophenanthroline disulfonate; SSC, standard saline citrate; TfR, transferrin receptor; FP1, ferroportin 1; FAS, ferrous ammonium sulfate; CP, cerebrospinal fluid ferroxidase; AM, acetoxymethyl ester.

given L-DOPA. Third, ferritin levels are significantly lower in several regions of postmortem brain of patients with PD who were treated with L-DOPA than those in the age-matched control patients (Dexter et al., 1990). These findings suggest the possible association of L-DOPA-induced changes in brain iron metabolism with the neurotoxicity of L-DOPA in patients with PD.

To explore this possibility, we investigated the effects of L-DOPA on the expression of some important proteins of brain iron metabolism, including transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferroportin 1 (FP1), as well as iron uptake in C6 glioma cells. The rat C6 glioma cell line was chosen for this study as a glial cellular model because it expresses TfR (Recht et al., 1990), DMT1 with or without iron-responsive element (IRE) [DMT1(+IRE) or DMT1(−IRE), respectively] (Lis et al., 2004), and ferroportin-1 (di Patti et al., 2004), and because of the importance of glial cells in iron homeostasis in the brain. Expression of TfR, DMT1, and FP1 was investigated because TfR and DMT1 are two major iron-influx proteins involved in iron uptake by the cells (Gunshin et al., 1997; Qian et al., 1997; Su et al., 1998), and FP1 (IREG1, MTP1, or Slc11a3) is a main iron exporter in mammals (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). The existence of these proteins in the brain has also been confirmed. Our data revealed that treatment of C6 cells with L-DOPA induced a significant increase in the expression of DMT1(−IRE), but not TfR, DMT1(+IRE), and FP1, and a remarkable increase in ferrous uptake by C6 glioma cells. The clinical relevance of the increased expression of the iron influx protein, and then cell iron accumulation induced by L-DOPA, needs to be investigated further.

Materials and Methods

Materials. Unless otherwise stated, all chemicals, including rabbit anti-human β-actin polyclonal antibody, were obtained from Sigma Chemical (St. Louis, MO). Bradford assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Hercules, CA), and TRIzol reagent, Dulbecco's modified Eagle's medium, and fetal bovine serum were from Invitrogen (Carlsbad, CA). ExpressHyb hybridization solution was obtained from Clontech (Mountain View, CA), and Prime-a-Gene labeling system was from Promega (Madison, WI). Micro Spin G-50 column, [α-^{32}P]dCTP, ECL Western blotting analysis system kit, anti-mouse secondary antibody, and 5% blocking reagent were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and streptomycin sulfate was from Invitrogen. Hybond-N membranes were products of GE Healthcare. Rabbit anti-rat DMT1(+IRE) and DMT1(−IRE1) polyclonal antibody and rabbit anti-mouse FP1 polyclonal antibody were purchased from Alpha Diagnostic (San Antonio, TX), and mouse anti-rat TfR monoclonal antibody was from BD Biosciences (San Jose, CA). Calcein acetoxymethyl ester (calcein-AM) was purchased from Invitrogen. Stock Fe^{2+} aqueous solutions (20 mM) were always prepared fresh as ferrous ammonium sulfate (FAS; Sigma Chemical) according to Picard et al. (2000) and Breuer et al. (2000).

C6 Glioma Cell Culture and Treatment. The rat C6 glioma cell line, obtained from the American Type Culture Collection (Manassas, VA), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (v/v), sodium penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere at 37°C and 5% CO_2. To determine the effects of L-DOPA on the expression of TfR, DMT1, FP1, and iron uptake, the rat C6 glioma cells were pretreated with different concentrations of L-DOPA (0–100 μM) for a given period, and then Northern blot and Western blot assays and measurement of quenching of calcein fluorescence were performed.

RNA Purification, Generation of Specific Probes, and Northern Blot Assay. Total RNA was isolated from C6 rat glioma cells pretreated with or without different concentrations of L-DOPA using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The polymerase chain reaction products corresponding to positions nucleotides 5 to 574 (M58040) of TfR, 1755 to 2592 (GenBank accession number AF008439) of DMT1(+IRE), 1697 to 2031 (GenBank accession number AF029757) of DMT1(−IRE), and 1298 to 1733 (GenBank accession number AF394785) of FP1 were used to generate the special ^{32}P-labeled probes. The RNA samples (30 μg) were subjected to electrophoresis on 1.2% formaldehyde-agarose gels, transferred to Hybond-N membranes with 10× standard saline citrate (SSC), and immobilized using a UV cross-linker (Fisher Scientific Co., Pittsburgh, PA). The blots were prehybridized at 65°C in ExpressHyb hybridization solution (Clontech) for 1 h and then hybridized overnight at 65°C in the same solution containing ^{32}P-labeled probes using the Prime-a-Gene labeling system. After three washes with 2× standard SSC containing 0.05% SDS at room temperature, the blots were washed three times in 0.1× SSC and 0.1% SDS with continuous shaking at 50 to 60°C. Radioactivity was then detected using a phosphorimager and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For normalization, the blot was stripped and reprobed with β-actin probe corresponding to position 474 to 736 of rat β-actin (GenBank accession number NM031144). The results were expressed as the ratio to β-actin.

Western Blot Analysis. Untreated C6 rat glioma cells and those treated with different concentrations of L-DOPA were washed with ice-cold phosphate-buffered saline (Invitrogen); homogenized with Tris buffer containing 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (pepstatin 1 μg/ml, aprotinin 1 μg/ml, and leupeptin 1 μg/ml); and then subjected to sonication using Soniprep 150 (MSE Scientific Instruments, London, UK) three times for 10 s each. After centrifugation at 10,000*g* for 15 min at 4°C, the supernatant was collected, and protein content was determined using the Bradford assay kit (Bio-Rad). Aliquots of the cell extract containing 40 μg of protein were diluted in 2× sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-PAGE on a 10% gel and subsequently transferred to a pure nitrocellulose membrane. After transfer, the membrane was blocked with 5% blocking reagent in Tris-buffered saline containing 0.1% Tween 20 overnight at 4°C. The membrane was rinsed in three changes of Tris-buffered saline/Tween 20, incubated in fresh washing buffer once for 15 min and twice for 5 min, and then incubated for overnight at 4°C with primary antibodies: rabbit anti-rat DMT1(+IRE), DMT1(−IRE1) polyclonal antibody, rabbit anti-mouse FP1 polyclonal antibody, 1:5000; mouse anti-rat TfR monoclonal antibody, 1:1000. After three washes, the membrane was incubated for 2 h in horseradish peroxidase-conjugated anti-rabbit or anti-mouse second antibody (1:5000) and developed using enhanced chemiluminescence (ECL Western blotting analysis system kit). The blots were detected using a Lumi-imager F1 workstation (Roche Molecular Biochemicals, Mannheim, Germany). The intensity of the specific bands was determined by densitometry with the use of LumiAnalyst 3.1 software (Roche Molecular Biochemicals). To ensure even loading of the samples, the same membrane was probed with rabbit anti-human β-actin polyclonal antibody at a 1:5000 dilution. In certain experiments, the cells were pretreated with 1 mM bathophenanthroline disulfonate (BP), an Fe^{2+} chelator, for 16 h at 37°C, and then expression of DMT1-IRE protein was determined.

Calcein Loading of the Cells and Iron Transport Assay. The cells were loaded with calcein-AM according to a method described previously (Ci et al., 2003). In brief, the cells were incubated with L-DOPA (0 or 10 μM) or BP (1 mM) in Dulbecco's modified Eagle's medium for 16 h and then washed twice with medium and incubated at a density of 6×10^5 cells/ml with 0.125 μM calcein-AM in serum-free medium for 5 min at 37°C. Excess calcein-AM on cell surface was

removed by three washes with Hanks' balanced salt solution, pH 7.4. Just before measurements, 100 μl of calcein-loaded cell suspension (approximately 6×10^5 cells) and 100 μl of HEPES were added to a 96-well plate. After initial baseline of fluorescence intensity was collected, FAS (4 μM, final concentration in incubation medium) was added to the plates. The fluorescence was measured with a Fluostar Galaxy fluorescence plate reader (BMG, Durham, NC) (λ_{ex} of 485 nm, λ_{em} of 520 nm, 37°C) equipped with excitation and emission probes directed to the bottom of the plate. The quenching of calcein fluorescence was recorded in every 5 min for 30 min. Data were normalized to the steady-state (baseline) values of fluorescence. To avoid the potential effects of intracellular transferrin on experimental results, the C6 rat glioma cells were suspended in Dulbecco's modified Eagle's medium/HEPES medium and maintained at 37°C for a minimum of 4 h to deplete intracellular store of transferrin before calcein loading of the cells.

Statistical Analysis. The difference between means was determined by one-way analysis of variance followed by a Student-Newman-Keuls test for multiple comparisons. The results were expressed as means ± S.E.M. A probability value of $P < 0.05$ was taken to be statistically significant.

Results

Effects of L-DOPA on TfR Expression in C6 Glioma Cells. We investigated the effects of L-DOPA on the expression of TfR mRNA and protein in C6 glioma cells. The cells grown on 75-cm^2 flask were treated with L-DOPA (0, 10, 30, and 100 μM, or 1, 5, 10, 30, 60, and 100 μM) for 16 h. Northern blot and Western blot analysis was then conducted. Figure 1, A and C, respectively, presents representative Northern blot (a signal mRNA band with expected molecular mass of ~5 kDa) and Western blot (a single band with a molecular mass of ~90 kDa). The relative values of TfR mRNA and protein are summarized in Fig. 1, B and D. The data demonstrated that L-DOPA did not induce any changes in the expression of TfR mRNA and protein.

Effects of L-DOPA on Expression of DMT1(+IRE) and DMT1(−IRE) mRNA and Protein in C6 Glioma Cells. To determine the effects of L-DOPA on the expression of two forms of DMT1 mRNAs and proteins, C6 glioma cells were treated with L-DOPA (10, 30, and 100 μM, or 1, 5, 10, 30, 60, and 100 μM), and then Northern and Western blot analyses were conducted. Cells not exposed to L-DOPA were used as controls. Figure 2, A and C, respectively, present representative Northern blots (a single major band with a molecular mass of ~4.4 kDa for DMT1(+IRE), 2.4 kDa for DMT1(−IRE), and 2 kDa for β-actin), and Fig. 2, E and G, presents representative Western blots (a single band with a molecular mass of ~56 kDa for two isoforms of DMT1 and ~45 kDa for β-actin). The results were in good agreement with the expected molecular masses based on published data (Conrad et al., 2000; Gambling et al., 2001). Figure 2, B, D, F, and H, show relative values of DMT1 mRNAs (B and D) and proteins (F and H). Treatment of C6 cells with different concentrations of L-DOPA did not induce any changes in the expression of DMT1(+IRE) mRNA or protein. No difference was found between relative values of DMT1(+IRE) mRNA (Fig. 2B) and protein (Fig. 2F). However, a significant increase in the expression of DMT1(−IRE) mRNA was found in cells treated with 10 and 30 μM L-DOPA. In addition, treatment with L-DOPA (1, 5, 10, and 30 μM) induced a significant increase in the expression of DMT1(−IRE) protein. The levels of DMT1(−IRE) mRNA and protein both were the highest in the cells treated with 10 μM L-DOPA and then decreased progressively with increasing concentrations of L-DOPA. Although the values of DMT1(−IRE) mRNA at 100 μM and protein at 60 and 100 μM L-DOPA are higher than those at 0 μM (the control), no significant differences were found among them. The data clearly revealed that the increase in DMT(−IRE) protein induced by L-DOPA treatment was in parallel with an increase in DMT(−IRE) mRNA in C6 glioma cells. The results also showed that treatment of C6 cells with 1 mM BP for 16 h did not induce any significant changes in the expression of DMT1(−IRE) protein (Fig. 3).

Effects of L-DOPA on Expression of FP1 Protein in C6 Glioma Cells. Ferroportin 1, a newly discovered transmembrane protein, is a major iron export protein in mammals (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). Decrease in the expression of FP1 might lead to a decrease in cell iron export and then iron accumulation in the cells. Therefore, we also investigated the effect of L-DOPA on the expression of FP1 protein in C6 glioma cells. The cells were treated with L-DOPA (0, 1, 5, 10, 30, 60, or 100 μM), and

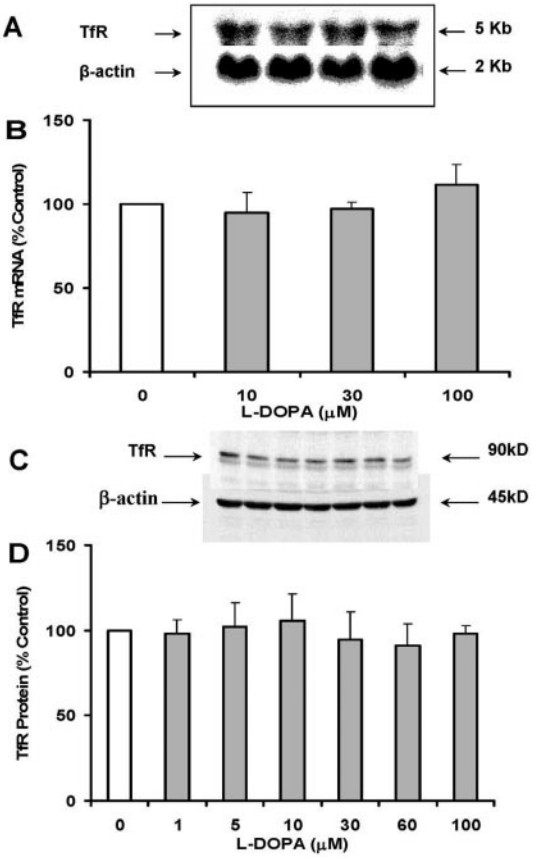

Fig. 1. Effects of L-DOPA on expression of TfR in C6 glioma cells. Total cellular RNA was isolated from C6 glioma cells that were pretreated with L-DOPA (0, 10, 30, or 100 μM) for 16 h. Northern blot was then performed. To confirm equal loading of RNA, each blot was rehybridized with ^{32}P-labeled probe for β-actin. In the case of TfR protein assay, the cells were pretreated with different concentrations of L-DOPA (0, 1, 5 10, 30 , 60, or 100 μM) for 16 h, and Western blot analyses were then performed as described under *Materials and Methods*. A, representative Northern blot (a signal mRNA band with molecular mass of ~5 kDa). B, the relative values of TfR mRNA expression. C, representative Western blot (a single band with a molecular mass of ~90 kDa). D, the relative values of TfR protein. B (mRNA) and D (protein) were normalized for β-actin. Data are means ± S.E.M. (percentage of control) from three independent experiments. No significant differences were found among the values.

Western blot analysis was then conducted. Figure 4A presents representative Northern blots (a single major band with a molecular mass of ~60 kDa for FP1 and 45 kDa for β-actin), and Fig. 4B shows the relative values of FP1 protein. No significant difference in expression of FP1 protein was found among the cells treated with or without different concentrations of L-DOPA.

Effects of L-DOPA and BP on Iron Uptake by C6 Glioma Cells. To confirm that the calcein method provides a valid measure of the ferrous uptake, a baseline signal was obtained from normal cells and those with no ferrous added cells (Fig. 5). This indicated that the fluorescence was steady in the 30-min recording. After stabilization of the fluorescence signal, FAS (4 μM, final concentration) was added and incubated with untreated C6 cells and those treated with L-DOPA (10 μM) or BP (1 mM). Because the levels of DMT1(−IRE) protein were the highest in the cells treated with 10 μM L-DOPA, this concentration was therefore used in this assessment. Results showed that FAS time-dependently quenched calcein fluorescence. There was no significant difference between cells treated with L-DOPA (L-DOPA group) and without L-DOPA (control group) for up to 15 min. The significant difference in quenching of fluorescence was observed between the two groups (control and L-DOPA) after incubating for 20 min or longer at 37°C (Fig. 5). This indicated that L-DOPA (10 μM) treatment could significantly increase ferrous uptake by C6 glioma cells. However, no significant difference was found between ferrous uptake by the cells treated with or without BP (Fig. 5).

Discussion

To our knowledge, this study is the first investigation of the effects of L-DOPA on the expression of iron influx proteins

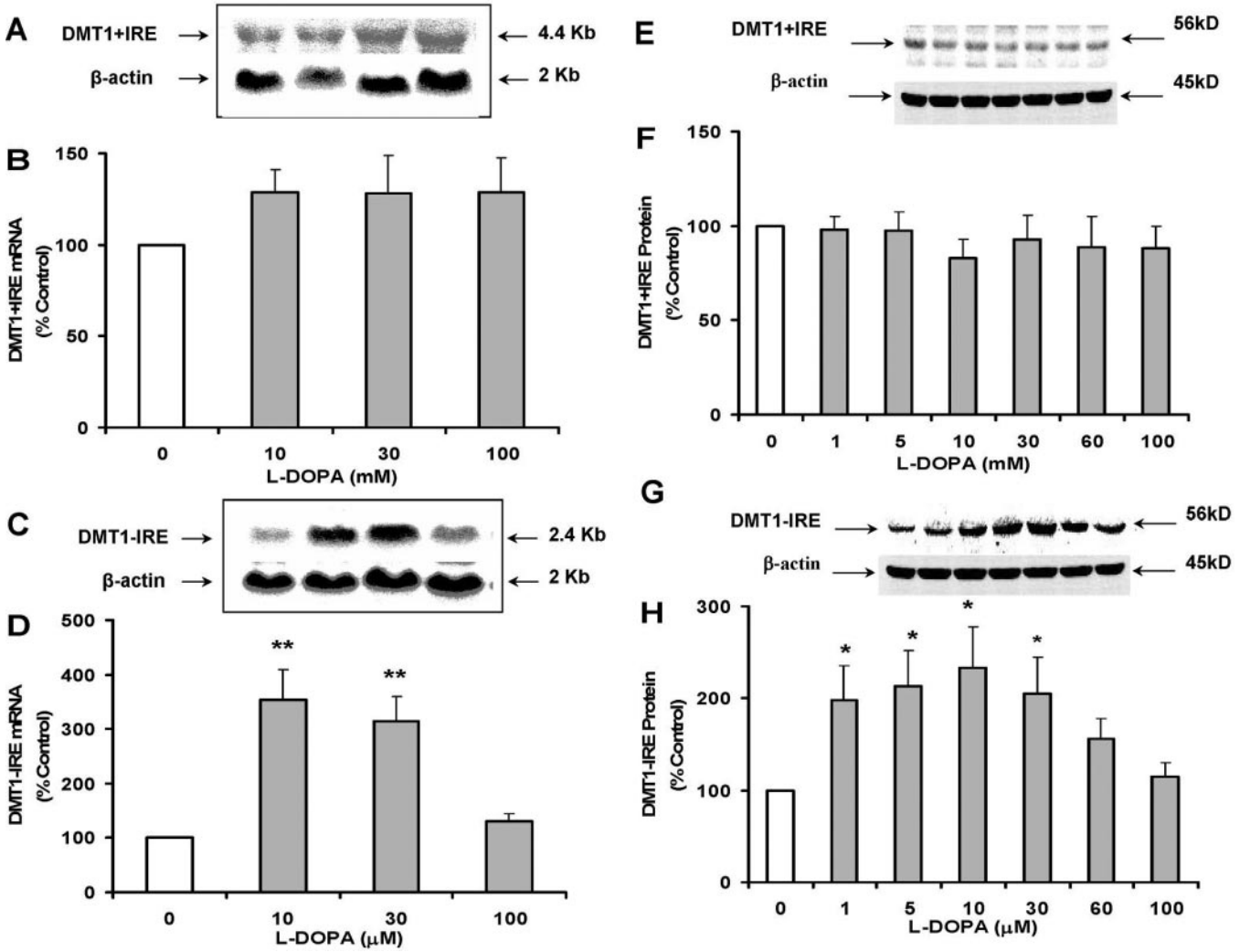

Fig. 2. Effects of L-DOPA on expression of DMT1(+IRE) and DMT1(−IRE) mRNA and protein in C6 glioma cells. The C6 glioma cells were pretreated with L-DOPA (0, 10, 30, and 100 μM) for 16 h, and total cellular RNA was isolated. Northern blot analyses were then performed. To confirm equal loading of RNA, each blot was rehybridized with ^{32}P-labeled probe for β-actin. Western blot was conducted using cells pretreated with different concentrations of L-DOPA (0, 1, 5, 10, 30, 60, and 100 μM) for 16 h as described under *Materials and Methods*. A, representative Northern blots of DMT1(+IRE) and β-actin (molecular masses of ~4.4 and 2 kDa, respectively). B, the relative values of DMT1(+IRE) mRNA expression. C, representative Northern blots of DMT1(−IRE) and β-actin (molecular masses of ~2.4 and 2 kDa, respectively). D, the relative values of DMT1(−IRE) mRNA expression. E, representative Western blots of DMT1(+IRE) and β-actin (molecular masses of ~56 and 45 kDa, respectively). F, the relative values of DMT1(+IRE) protein expression. G, representative Western blots of DMT1(−IRE) and β-actin (molecular masses of ~56 and 45 kDa, respectively). H, the relative values of DMT1(−IRE) protein expression. Data are means ± S.E.M. (percentage of control) from three independent experiments. *, $P < 0.05$ versus the control (0 μM L-DOPA).

(TfR and DMT1), iron efflux protein (FP1), and iron uptake in C6 glioma cells. Our data show that treatment of C6 cells with L-DOPA did not induce any changes in the expression of TfR and DMT1(+IRE) mRNA and protein and ferroportin 1 protein. However, a significant increase in the expression of DMT1(−IRE) mRNA and protein was found in cells treated with 10 and 30 μM L-DOPA (mRNA) and with 1, 5, 10, and 30 μM L-DOPA (protein), respectively. The increase in DMT(−IRE) protein induced by L-DOPA treatment was in parallel with the increase in DMT(−IRE) mRNA. By plotting the values of DMT1(−IRE) mRNA and protein against one another, a highly significant correlation was found (r = 0.927, $P < 0.001$). This implied that DMT1(−IRE) gene expression is regulated by L-DOPA at the transcriptional level.

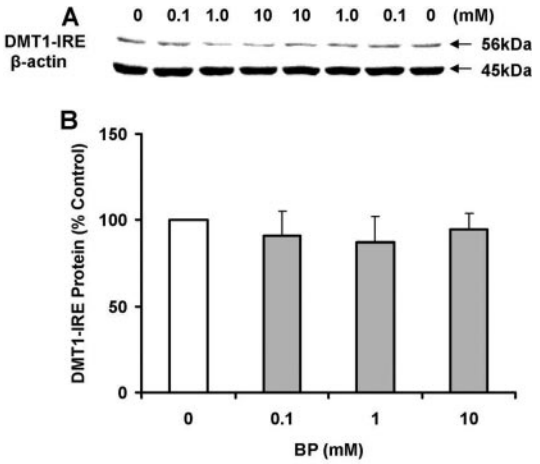

Fig. 3. Effects of BP on expression of DMT1(−IRE) protein in C6 glioma cells. Western blot was performed using cells pretreated with BP (0 or 1 mM) for 16 h as described under *Materials and Methods*. A, representative Western blots of DMT1(−IRE) and β-actin. B, the relative values of DMT1(−IRE) protein expression. Data are means ± S.E.M. (percentage of control) from three independent experiments. There is no significant difference in the levels of DMT1(−IRE) protein of C6 glioma cells pretreated with or without BP.

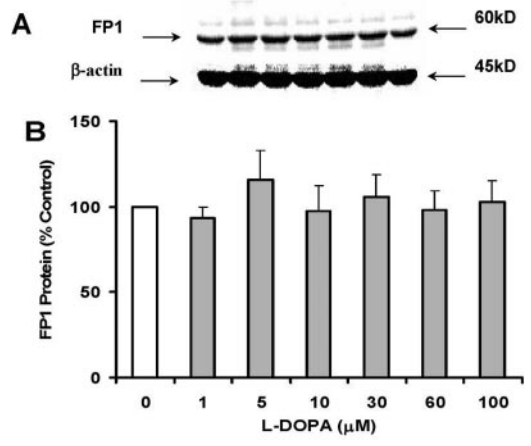

Fig. 4. Effects of L-DOPA on the expression of FP1 protein in C6 glioma cells. The C6 glioma cells were pretreated with L-DOPA (0, 1, 5, 10, 30, 60, and 100 μM) for 16 h, and Western blot analyses were then performed as described under *Materials and Methods*. Values of protein expression were normalized for β-actin, and data were means ± S.E.M. (percentage of control) of three independent experiments. A, representative Western blots of FP1 and β-actin (molecular masses of 60 and 45 kDa, respectively). B, the relative values of FP1 protein expression. No significant difference in FP1 protein expression was found between the cells treated with or without L-DOPA.

The levels of DMT1(−IRE) protein peaked in cells treated with 10 μM L-DOPA and then decreased progressively with increasing concentrations of L-DOPA. Further study demonstrated that treatment of cells with 10 μM L-DOPA induced a significant increase in ferrous uptake by C6 glioma cells.

DMT1 (DCT-1 or NRAMP2) is a widely expressed membrane protein (Fleming et al., 1997; Gunshin et al., 1997). It is responsible for the uptake of a broad range of divalent metal ions (Fleming et al., 1997; Gunshin et al., 1997; Tandy et al., 2000). The existence of this protein in the brain has been well determined (Burdo et al., 2001; Ke et al., 2004). In general, DMT1(+IRE) is predominantly expressed by epithelial cell lines, whereas DMT1(−IRE) is expressed by blood cell lines (Canonne-Hergaux et al., 1999, 2001). The functions of DMT1(+IRE) and DMT1(−IRE) have not been completely understood (Picard et al., 2000). However, available data support the notion that DMT1(−IRE), rather than DMT1(+IRE), is the entity responsible for transmembrane transport of the iron released from transferrin to the early endosomal lumen (Touret et al., 2003). Recent studies also inferred that DMT1(−IRE) is found predominantly in three compartments: the plasma membrane, early/recycling endosomes, and the endoplasmic reticulum (Touret et al., 2003). In the present study, we found a significant increase in the expression of DMT1(−IRE) as well as iron uptake in C6 glioma cells treated with L-DOPA. It implies that L-DOPA has a role in stimulating the expression of DMT1(−IRE) and then inducing a significant increase in cell iron uptake. The findings suggest that L-DOPA-induced changes in iron metabolism might be associated with some side effects of L-DOPA treatment of PD.

At present, it is unknown how L-DOPA regulates DMT1(−IRE) expression. L-DOPA, as a catechol compound, is a chelator for divalent metals. Therefore, it is possible that the increase in DMT1(−IRE) protein and consequently iron uptake-induced L-DOPA might be caused by the decreased intracellular iron resulting from the chelating effect of L-DOPA. To determine this possibility, we investigated the effects of BP, a chelator of divalent metals (Fe^{2+}), on the expression of DMT1(−IRE) protein and iron uptake in the cells. The results showed that treatment of C6 cells with 1 mM BP for 16 h did not induce any significant changes in the

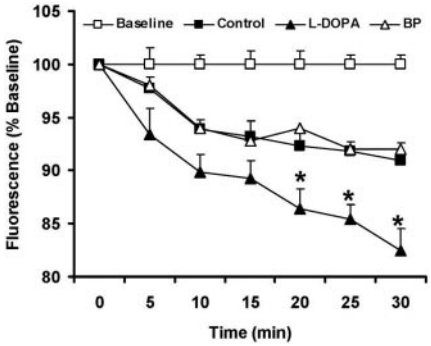

Fig. 5. Effects of L-DOPA and BP on iron uptake by C6 glioma cells. After the initial baseline (fluorescence intensity) was collected, FAS (final concentration is 4 μM in incubation medium) was added and incubated with the cells that were pretreated with 0 μM (control), 10 μM L-DOPA (L-DOPA), or 1 mM BP (BP). The quenching of calcein fluorescence by iron was measured every 5 min for 30 min. The fluorescence assay for iron uptake by C6 cells was performed in duplicate for three independent experiments. The data represent means ± S.E.M. (percentage of baseline). *, $P < 0.05$ versus the control group.

expression of DMT1(−IRE) protein and iron uptake. These findings are in agreement with the ones reported by Barisani et al. (1995), who found no effect of the Fe^{2+} chelator ferrozine (1 mM) on ^{55}Fe uptake by rat hepatocytes. This implies that the increased expression of the DMT1(−IRE) protein and cell iron accumulation, induced by L-DOPA, is not caused by the chelating effect of L-DOPA. To understand the mechanisms involved in the increase in DMT1(−IRE) expression, further studies are needed.

Cell iron balance depends on iron uptake and iron efflux (Qian and Wang, 1998). Accumulated evidence shows that FP1/CP or FP1/hephaestin systems might play a key role in iron efflux from cells in the brain (Qian and Wang, 1998; Qian and Ke, 2001; Qian et al., 2002; Ke and Qian, 2003). The effect of L-DOPA on the expression of these iron efflux proteins is another important issue that needs to be addressed. A clinical study (Boll et al., 1999) demonstrated that L-DOPA can significantly affect brain CP and that cerebrospinal fluid CP in L-DOPA-treated patients with PD is significantly higher than the one observed in patients with PD who were not given L-DOPA. CP is a key protein in brain iron metabolism and is widely believed to have a role in iron release from brain cells. However, as an enzyme, CP itself is unlikely to be a membrane iron transporter (Vulpe et al., 1999; Anderson et al., 2002). In addition to CP, FP1 has recently been suggested as a key player in iron release from brain neurons and in iron transport across the basolateral membrane of the blood-brain barrier (Qian and Ke, 2001; Qian and Shen, 2001; Qian et al., 2002). This newly discovered transmembrane iron export protein might interact with CP or its homolog hephaestin in the process of iron release from brain cells. Recent studies have demonstrated that FP1 is particularly abundant in the brain (Burdo et al., 2001; Jiang et al., 2002; Jeong and David, 2003; Wu et al., 2004). It strongly supports the hypothesis that this protein may function to export iron from brain cells, playing a physiological role in brain iron homeostasis. In this study, we did not measure CP content but found that L-DOPA treatment had no effect on FP1 expression in the cells. This implies that L-DOPA has no effect on cellular iron efflux but can increase the expression of iron influx protein DMT1(−IRE) and iron influx into the cells. In this case, cell iron will overaccumulate. It might be one of the causes for the neurotoxicity of L-DOPA.

Our study provides initial and important evidence for the association of the L-DOPA-induced changes in iron metabolism with the side effects of L-DOPA treatment in PD. The data we present show that L-DOPA is maximally active at the concentration of 10 μM in vitro. However, it is unknown at present whether this concentration is likely to be reached in the brain of patients with PD treated with L-DOPA, because a number of factors affect the concentration of L-DOPA in the brain in vivo, including decarboxylation of L-DOPA, the speed of which is very rapid. It should therefore be pointed out that the clinical relevance of the increased expression of the iron influx protein and then the cell iron accumulation induced by L-DOPA needs to be investigated further.

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Address correspondence to: Dr. Zhong Ming Qian, Department of Applied Biology and Chemistry Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong. E-mail: bczmqian@polyu.edu.hk