Changes in iron-regulatory gene expression occur in human cell culture models of Parkinson’s disease

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Background: Neuronal iron accumulation is thought to be relevant to the pathogenesis of Parkinson’s disease (PD), although the mechanism remains elusive. We hypothesized that neuronal iron uptake may be stimulated by functional mitochondrial iron deficiency.

Objective: To determine firstly whether the mitochondrial toxin, 1-methyl-4-phenylpyridinium iodide (MPP+), results in upregulation of iron-import proteins and transporters of iron into the mitochondria, and secondly whether similar changes in expression are induced by toxins with different mechanisms of action.

Methods: We used quantitative PCR and Western blotting to investigate expression of the iron importers, divalent metal transporter (DMT1) (Lee et al., 1998), transferrin receptor 1 and 2 (TfR1 and TfR2) and mitoferrin-2 and the iron exporter ferroportin in differentiated SH-SY5Y cells exposed to three different toxins relevant to PD, MPP+, paraquat (a free radical generator) and lactacystin (an inhibitor of the ubiquitin–proteasome system (UPS)).

Results: MPP+ resulted in increased mRNA and protein levels of genes involved in cellular iron import and transport into the mitochondria. Similar changes occurred following exposure to paraquat, another inducer of oxidative stress. Lactacystin also resulted in increased TfR1 mRNA levels, although the other changes were not found.

Conclusion: Our results support the hypothesis of a functional mitochondrial iron deficit driving neuronal iron uptake but also suggest that differences exist in neuronal iron handling induced by different toxins.

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1. Introduction

Increase in intraneuronal iron is thought to be relevant to the pathogenesis of Parkinson’s disease (PD) (Kaur and Andersen, 2004); The mechanism remains elusive but may involve changes in expression of iron transport proteins, including the iron importers, divalent metal transporter (DMT1) (Lee et al., 1998), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2), possibly responsible for transporting transferrin-bound iron into mitochondria (Mastroberardino et al., 2009), and mitoferrin-2 (Mfrn2), a protein involved in mitochondrial iron uptake in non-erythroid cells (Richardson et al., 2010), and the exporter ferroportin (FPN) (Abbond and Haile, 2000). DMT1 has two isoforms, one with an iron-response element (IRE), DMT1 (+IRE), and one without, DMT1 (–IRE).

A variety of toxin-based cell culture models of PD mimic the various abnormalities that have been implicated in the pathogenesis of PD: 1-methyl-4-phenylpyridinium iodide (MPP+) inhibits mitochondrial function (Kalivendi et al., 2004, 2003); paraquat is a free radical generator (Dinis-Oliveira et al., 2006); and lactacystin is a potent and specific inhibitor of the ubiquitin–proteasome system (UPS) (Zhang et al., 2005). The protective effects of iron chelation against each of these toxins has been demonstrated in a variety of models (Ayaki et al., 2005; Molina-Holgado et al., 2008; Santiago et al., 1997; Xu et al., 2008; Zhang et al., 2005; Zhu et al., 2007).

Oxidative damage to mitochondrial iron–sulphur clusters may result in mitochondrial functional iron deficit thereby acting as a
stimulus for neuronal iron accumulation (Mastroberardino et al., 2009; Paradkar et al., 2009). Previous studies in rat cerebellar granule cells and human neuroblastoma cells have demonstrated an increase in TR1 protein expression following exposure to MPP+ (Kalivendi et al., 2003). Postmortem studies have demonstrated increased [125I]Tf(Fe2) binding in the striatum of PD patients (Fauci-heux et al., 1995). There is increased expression of DMT1 (+IRE) in C6 cells treated with 6-hydroxydopamine (6-OHDA) at both the protein and mRNA level (Song et al., 2007) and increased protein in the ventral mesencephalon of MPTP-treated mice (Salazar et al., 2008). However, these findings contrast with those in a murine cell line exposed to MPP+ (Zhang et al., 2008). Increased expression of DMT1 (+IRE) protein has also been found in human parkinsonian nigra (Salazar et al., 2008). There is a reduction in FPN protein and mRNA levels in the substantia nigra of 6-OHDA-treated rats (Wang et al., 2007a). Additionally, it has been shown that in nigral dopaminergic cells of rotenone-treated rats there are increased levels of transferrin and TRR2 (Mastroberardino et al., 2009).

Although partially conflicting and incomplete, these data suggest that altered expression of genes involved in neuronal iron homeostasis occurs in sporadic PD and in animal and cell culture models, and that regulation may occur at the transcriptional level. Here we systematically investigate expression of DMT1, TfR1, TfR2, FPN, Mfrn2 and DMT+/-IRE. (FAM)-labeled TfR2 (Hs00162690_m1) and Mfrn2 (Hs00377907_m1) primers were purchased from Applied Biosystems (Warrington, Cheshire, UK). Primers for the remaining sequences were designed using Roche Universal ProbeLibrary Assay Design (Roche). In this system probes are labeled 5’–terminal with fluorescein (FAM) and 3’–proximal with a dark quencher dye. The following primers and probes were used: DMT1 (+IRE) forward 5’-catgccaagatgtcc-3’, reverse 5’-ttgactgttgctgtcctgatataga-3’, probe 17; DMT (–IRE) forward 5’-ctctacctcctccatctgctta-3’, reverse 5’-cccatctccatctcctcgttc-3’, probe 43; ferroportin forward 5’-cattcgagctgggagggt-3’, reverse 5’-ctaatctggtacttgcctcct-3’, probe 44; TR1 forward 5’-tggagaaaa-tttggtcggt-3’, reverse 5’-cccagttgctgtcctgatataga-3’, probe 61. Reactions were carried out on a Biorad iCycler. Each reaction was run in triplicate with 1 μl sample in a total volume of 20 μl. Amplification and detection were performed with the following conditions: an initial hold at 95 °C for 10 s followed by 50 cycles at 95 °C for 15 s and 60 °C for 10 s. Gene expression was normalized to 18 s expression run in triplicate concurrently. All samples were analyzed in triplicate on triplicate samples from four separate experiments. Statistical significance was determined using one-way analysis of variance in SPSS.

2.4. LDH-release assay

To assess the cytotoxicity of the toxins under our experimental conditions, LDH release was measured in treated cells grown in 96-well plates. Cell culture medium (50 μl) was used to analyze the LDH activity by measuring the oxidation of NADH at 450 nm as described in the manufacturer’s protocol (Promega). The remaining cells were lysed and LDH activity similarly measured to allow the percentage of cell death to be calculated. Each assay was performed a minimum of six times in quadruplicate.

2.5. Measurement of reactive oxygen species

SH-SYSY cells were seeded into 96 well plates. After 48 h of treatment with MPP+, lactacystin and pararquat (for 6, 12, 24 and 48 h), medium was removed and cells were incubated with 10 μM 2,7’-Dichlorodihydrofluorescein diacetate (DCFDA) (Sigma–Aldrich) in medium for 30 min. Cells were then washed three times with PBS after which the fluorescence was measured at Ex:485 nm and Em:535 nm. The LDH assay was used to determine the proportion of surviving cells. Each assay was performed with a minimum of eight repeats on two separate occasions. Statistical significance was determined using one-way analysis of variance in SPSS.

2.6. Total RNA extraction and quantitative PCR

Total RNA was extracted using GenElute (Sigma–Aldrich) and treated with DNA-free (Ambion, Huntingdon, UK) according to the manufacturer’s instructions. Total RNA was reverse transcribed in a 50 μl reaction using cDNA Archive Kit (Ambion). Quantitative PCR was employed to detect changes of ferroportin, TR1, TR2, Mfrn2 and DMT+/– IRE. (FAM)-labeled TR2 (Hs00162690_m1) and Mfrn2 (Hs00377907_m1) primers were purchased from Applied Biosystems (Warrington, Cheshire, UK). Primers for the remaining sequences were designed using Roche Universal ProbeLibrary Assay Design (Roche). In this system probes are labeled 5’–terminal with fluorescein (FAM) and 3’–proximal with a dark quencher dye. The following primers and probes were used: DMT1 (+IRE) forward 5’-catgccaagatgtcc-3’, reverse 5’-ttgactgttgctgtcctgatataga-3’, probe 17; DMT (–IRE) forward 5’-ctctacctcctccatctgctta-3’, reverse 5’-cccatctccatctcctcgttc-3’, probe 43; ferroportin forward 5’-cattcgagctgggagggt-3’, reverse 5’-ctaatctggtacttgcctcct-3’, probe 44; TR1 forward 5’-tggagaaaa-tttggtcggt-3’, reverse 5’-cccagttgctgtcctgatataga-3’, probe 61. Reactions were carried out on a Biorad iCycler. Each reaction was run in triplicate with 1 μl sample in a total volume of 20 μl. Amplification and detection were performed with the following conditions: an initial hold at 95 °C for 10 s followed by 50 cycles at 95 °C for 15 s and 60 °C for 10 s. Gene expression was normalized to 18 s expression run in triplicate concurrently. All samples were analyzed in triplicate on triplicate samples from four separate experiments. Statistical significance was determined using one-way analysis of variance in SPSS.

2.7. Protein extraction and Western blot analysis of protein levels

Cells were lysed for protein extraction at 6, 12, 24 and 48 h following exposure to the toxins. Cells were washed with ice-cold PBS and protein extracted with NET-Triton buffer (150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.4, 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma–Aldrich) according to the manufacturer’s instructions. Protein level was estimated using the bicinchoninic acid (BCA) assay. 20 μg was loaded into each well for Western blotting.
The proteins were resolved by SDS/PAGE (10% gels) and blotted onto PVDF membranes. Membranes were washed with Tris-buffered saline (140 mM NaCl, 50 mM tris/HCl, pH7.2) containing 0.1% Tween 20, 5% skimmed milk and 2% BSA to block the non-specific protein binding. Membranes were incubated with primary antibody against TR1 (abcam, Cambridge UK, ab65831), TR2 (abcam ab84287), DMT1 (both isoforms) (Autogen Biochecl, Wiltshire, UK NRAMP-22A), Mfrn2 (abcam, Cambridge UK, ab74681) and FPN (antibody kind gift from David Haile) in Tris-buffered saline (140 mM NaCl, 50 mM tris/HCl, pH7.2) containing 0.1% Tween 20, 5% skimmed milk and 2% BSA overnight at 4 °C, washed three times and then incubated with horseradish-peroxidase-conjugated secondary antibody for 1 h at room temperature. To correct for variation in protein levels, the same membrane was probed with rabbit secondary antibody for 1 h at room temperature. To correct for variation in protein levels, the same membrane was probed with rabbit anti-human β-actin antibody (abcam) at a 1:5000 dilution. The protein bands were detected using the ECL method (Amersham Biosciences, Little Chafont, Buckinghamshire, UK) and then analyzed through scanning densitometry by a BioRad Fluor-S Multimager. Results are based on a mean of a minimum of six different samples. Statistical significance was determined using one-way analysis of variance in SPSS.

3. Results

3.1. Changes in gene and protein expression

We found that MPP⁺ resulted in a significant increase in mRNA levels for the iron import proteins, TR1 and DMT1 (+IRE) as well as the presumed mitochondrial iron transporters, TR2 and Mfrn2. (Fig. 1). We found a non-significant increase in the level of mRNA for the iron export protein FPN (p = 0.051). In addition there was a 10 fold reduction in mRNA level for DMT1 (−IRE). The changes observed were largely paralleled by changes in protein expression with the exception of DMT, where no significant change in expression was observed, probably due to the antibody recognizing both isoforms of DMT. In addition protein levels of mitoferrin-2 reduced significantly over 48 h in contrast to the increase in mRNA. Finally we demonstrated increased levels of transferrin into MPP⁺-treated cells over 48 h, although this failed to reach significance (Fig. 1f).

We were interested to see whether similar changes in mRNA occurred with another free-radical generating toxin, paraquat, and found similar changes in both isoforms of DMT and TR1, with more modest but significant upregulation of TR2 and Mfrn2; in addition there was a slight reduction in mRNA for FPN (Fig. 2). Similar changes in protein expression to those following MPP⁺ were also observed. However, while MPP⁺ treatment resulted in a significant increase in TR1 and TR2 protein expression at 48 h, protein levels peaked at 12 h of treatment with PQT. The most notable difference between MPP⁺ and PQT treatment was a marked reduction in mitoferrin-2 expression following 12 h exposure to PQT with recovery at 24 and 48 h.

In order to investigate whether similar changes occurred with a toxin used to model PD with a different mechanism of action, differentiated cells were exposed to lactacystin, a potent and specific inhibitor of the UPS. This resulted in a significant reduction in mRNA level for all the proteins investigated within either 12 or 24 h, with the exception of mitoferrin-2, although there was a significant 1.8 fold increase in TR1 mRNA at 48 h (Fig. 3). Western blotting revealed no change in total DMT and an increase in Mfrn2 and FPN at 48 h. There was no increase in transferrin.

Finally to determine whether the differences observed between the responses to the toxins could be explained by different degrees of oxidative stress induced we measured ROS using the DCFDA assay, which primarily measures hydrogen peroxide production. This demonstrated a significant increase in ROS with all three toxins compared with vehicle, although it was much greater with MPP⁺ than with paraquat or lactacystin (Fig. 4).

4. Discussion

In this study we investigated MPP⁺, a toxin associated with oxidative stress in a human cell culture model to mimic PD, and demonstrated expression change of proteins involved in neuronal iron import and transport of iron into mitochondria. These findings support those of studies involving animal models or rodent cell lines in which increased expression of DMT1 (+IRE) (Salazar et al., 2008), TR1 (Kalivendi et al., 2003; Shang et al., 2004) and TR2 (Mastroberardino et al., 2009) have been demonstrated following exposure to mitochondrial inhibitors, MPP⁺ or rotenone. Although we have demonstrated changes in iron regulatory proteins in our study, we do not know whether this translates into an actual change in cellular iron content. Nevertheless our systematic data on the mechanism of iron import support the hypothesis that mitochondrial functional iron deficit acts as a stimulus for iron import, which may result in neuronal iron overload and contribute to neuronal death.

MPP⁺ is an inhibitor of complex I, resulting in production of reactive oxygen species (Fiskum et al., 2003; Hartley et al., 1994; Przedborski and Vila, 2003), which are known to cause oxidation of thiols in cysteine residues (Mastroberardino et al., 2009) and carboxylation of complex I itself (Keeney et al., 2006). We demonstrated intracellular production of hydrogen peroxide following MPP⁺ exposure using the DCFDA assay, suggestive of mitochondrial dysfunction. Although maximal levels of ROS were seen at 48 h, it is likely that mitochondrial dysfunction occurred at earlier time points, resultant radical production being ameliorated by intracellular quenchers. It has been suggested that oxidation of Fe–S clusters within mitochondrial proteins results in impaired utilization of iron (Mastroberardino et al., 2009). We hypothesized that the mitochondrial functional iron deficiency induced by MPP⁺ would result in upregulation of iron-import proteins and transporters of iron into the mitochondria, as has been suggested to occur in erythroid and non-erythroid cells following inhibition of heme synthesis or frataxin-deletion (Paradkar et al., 2009; Richardson et al., 2010). Our finding of increased TR1, DMT1 (+IRE), TR2 and Mfrn2 mRNA levels following MPP⁺ exposure, largely paralleled by protein levels and increase in transferrin supports this hypothesis. Interestingly we found a marked reduction in mitoferrin-2 protein level following MPP⁺ exposure. It is likely that this reduction results in persistently reduced mitochondrial iron delivery even in the presence of increased cytosolic iron (Paradkar et al., 2009), thereby perpetuating mitochondrial dysfunction. Although it has been well demonstrated that many factors influence the correlation between measured mRNA and protein levels (Guo et al., 2008; Maier et al., 2009), the divergent results of mRNA and protein levels of mitoferrin–2 raise interesting questions about the regulation of mitoferrin–2 expression, which is as yet poorly understood. One possibility is regulation of protein degradation resulting in altered half-life as has been suggested for mitoferrin–1 in erythrocyte cells (Paradkar et al., 2009).

We predicted that similar changes in expression of these genes and proteins would result after exposure to other toxins associated with oxidative stress, such as paraquat, and demonstrated that this was the case. Paraquat acts as a free radical generator (Ayaki et al., 2005; Dinis-Oliveira et al., 2006) and has been demonstrated to result in apoptosis in SH-SY5Y cells (Gonzalez-Polo et al., 2007a,b). One mechanism by which the mRNA changes may occur is increased binding of iron response proteins (IRPs) to the 3’UTR IREs of TR1 and DMT1 (+IRE), increasing the cytoplasmic stability of the transcripts (Mullner and Kuhn, 1988; Mullner et al., 1989).
IRP1 functions as an aconitase when bound to a 4Fe–4S cluster. In situations of oxidative stress, Fe–S disassembly directs IRP1 back to its high IRE affinity form (Volz, 2008). It has previously been demonstrated that paraquat increases binding of IRP1 to IREs (Ayaki et al., 2005). Our finding of mRNA level for both DMT (+IRE) and TfR1 being highest at 48 h following exposure to both MPP⁺ and paraquat, paralleling the time of maximal ROS production, supports this hypothesis. However, interestingly, we found increase in TfR1 protein level at earlier time points following exposure to both MPP⁺ and paraquat, suggesting that other mechanisms exist to control TfR1 protein expression under these conditions. These may include translational stimulation as has been shown to be the case following sustained exposure to low levels of hydrogen peroxide in other cell culture systems (Andriopoulos et al., 2007).

In contrast to DMT (+IRE), we found a significant reduction in the level of mRNA for the non-IRE isoform of DMT in response to MPP⁺ and paraquat. This finding is consistent with a reduction in DMT (–IRE) protein found in the substantia nigra of PD patients in a post mortem study (Salazar et al., 2008). This significant reduction in DMT (–IRE) mRNA may explain the lower expression of total DMT protein at the 24 and 48 h time points in MPP⁺ treated samples and the unchanged total DMT protein expression in PQT treated samples. DMT1 (+IRE) has been found to localize to the nuclei of neurons as well as the cell surface, suggesting that it may have a role other than iron import in neuronal cells (Roth et al., 2000). In contrast, DMT1 (+IRE) localizes to the cell surface of neurons as well as within vesicles within dendrites and axons, consistent with a role in iron uptake (Roth et al., 2000). Our finding that

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**Fig. 1.** Exposure of differentiated SH-SY5Y cells to MPP⁺ resulted in a significant increase in mRNA levels of TfR1 (a), DMT1 (+IRE) (b), TfR2 (c) and Mfrn2 (d). There was a significant reduction in mRNA level for DMT1 (–IRE) (b) and no significant change in mRNA for ferroportin (e). These changes were accompanied by similar changes in protein level, with the exception of total DMT and mitoferrin-2. There was also a non-significant increase in transferrin protein level (f) (*p < 0.05; **p < 0.005).
the two isoforms of DMT are differentially modulated in response to toxin exposure supports the notion that they have different functions in neuronal cells.

TfR2 is thought to act as a transporter of iron into mitochondria (Mastroberardino et al., 2009). The mRNA sequence of TfR2 does not contain an IRE, and therefore the mechanism by which levels are increased remains undetermined. Nevertheless we have demonstrated a significant increase in TfR2 mRNA levels following complex I inhibition with MPP+, and to a lesser extent following paraquat exposure, paralleled by protein increase, which is in keeping with a previous demonstration of an increase in nigral dopaminergic TfR2 protein expression in rotenone-treated rats (Mastroberardino et al., 2009). It has previously been shown in SH-SYSY cells that ferroportin protein levels are upregulated in response to increasing cellular iron content, and that this is associated with increased cell survival (Aguirre et al., 2005) However, the opposite effect of iron loading has been found in rat PC12 cells (Chen et al., 2005), implying that there may be important species and cell type differences in neuronal iron homeostasis. We found non-significant increases in ferroportin mRNA and protein level after exposure to MPP+, compatible with a protective response to iron loading. It has been suggested that ferroportin expression is down-regulated post-transcriptionally via 5′-IRP1/IRE interaction in response to inflammatory stimuli (Liu et al., 2002). Oxidative stress results in increased IRP1 activity and subsequent repression of mRNA translation (Volz, 2008). Our finding of a possible change

**Fig. 2.** A similar pattern of change in mRNA levels was seen following exposure of differentiated SH-SYSY cells to 300 µM paraquat, with a significant increase in mRNA levels for TfR1 (a), DMT1 (+IRE) (b), TfR2 (c) and Mfrn2 (d), and a significant reduction in mRNA level for DMT1 (−IRE) (b). There was a modest but significant decrease in mRNA for ferroportin (e) (‘p < 0.05; ‘‘p < 0.005). There were similar changes in protein level, with the exception of total DMT and mitoferrin-2. There was also a non-significant increase in transferrin protein level (f) (‘p < 0.05; ‘‘p < 0.005).
in mRNA as well as protein level is indicative of regulation of expression at a transcriptional level in addition to these possible translational mechanisms.

Disruption of the UPS is thought to be relevant not only to some familial forms of PD, but also to sporadic disease (McNaught et al., 2003). Lactacystin is a potent and specific UPS inhibitor which has been shown to result in ubiquitin and \( \alpha \)-synuclein positive aggregates in SH-SY5Y cells (Lev et al., 2006) and has been used to model PD in animal studies (McNaught et al., 2004). The mechanisms of action of MPP\(^+\) and paraquat have also been suggested to involve inhibition of the UPS (Domingues et al., 2008; Sun et al., 2007). However, we found that the pattern of change in mRNA and protein levels was different following UPS inhibition with lactacystin, which may reflect a lower induction of oxidative stress, although it has been proposed that lactacystin results in accumulation of oxidatively damaged proteins resulting in cell death (Zhang et al., 2005). We found that lactacystin resulted in initial reduction in mRNA levels for all of the proteins investigated other than Mfrn2, which may represent a down-regulation of transcription following disruption of normal protein trafficking induced by inhibition of the UPS. We also found increased expression of mitoferrin-2 and ferroportin proteins at 48 h; otherwise there was no change in protein expression (\( p < 0.05; **p < 0.005 \)).

Fig. 3. Exposure of differentiated SH-SY5Y cells to 20 \( \mu \)M lactacystin resulted in reduced levels of mRNA for all of the proteins investigated other than mitoferrin-2 (d): TIR1 (a), DMT1 (+IRE) (b), DMT1 (−IRE) (b), TIR2 (c), and FPN (e). However at 48 h there was a significant increase in mRNA level for TIR1 (a). There was a significant increase in mitoferrin-2 and ferroportin protein level at 48 h; otherwise there was no change in protein expression (\( p < 0.05; **p < 0.005 \)).

C.B. Carroll et al. / Neurochemistry International 59 (2011) 73–80
therefore result in increased levels of IRP2 with subsequent stabilization of mRNA for the TfR1.

5. Conclusions

In conclusion we have demonstrated in differentiated human neuroblastoma cells that the mitochondrial complex I inhibitor, MPP+, results in expression change for proteins involved in cellular iron import and transport of iron into the mitochondria, as well as an increase in transferrin, supportive of the hypothesis of a functional mitochondrial iron deficit driving neuronal iron uptake. Similar changes occur following exposure to paraquat, another inducer of oxidative stress. Although lactacystin, an inhibitor of the UPS, also resulted in increased TfR1 mRNA levels, the other changes were not found suggesting that there are significant differences in neuronal iron handling induced by the different toxins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2011.05.006.

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