Published in final edited form as:

Free Radic Biol Med. 2009 March 1; 46(5): 593–598. doi:10.1016/j.freeradbiomed.2008.11.012.

Glutathione depletion in immortalized midbrain-derived dopaminergic neurons results in increases in the labile iron pool: Implications for Parkinson's disease

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Abstract

Glutathione depletion is one of the earliest detectable events in the Parkinsonian substantia nigra (SN), but whether it is causative for ensuing molecular events associated with the disease is unknown. Here we report that reduction in levels of glutathione in immortalized midbrain-derived dopaminergic neurons results in increases in the cellular labile iron pool (LIP). This increase is independent of either iron regulatory protein/iron regulatory element (IRP/IRE) or hypoxia inducible factor (HIF) induction but is both H₂O₂ and protein synthesis-dependent. Our findings suggest a novel mechanistic link between dopaminergic glutathione depletion and increased iron levels based on translational activation of TfR1. This may have important implications for neurodegeneration associated with Parkinson's disease in which both glutathione reduction and iron elevation have been implicated.

Keywords

Glutathione depletion; Labile iron pool; Dopaminergic; Parkinson's disease; Transferrin receptor

Parkinson's disease (PD) is characterized by selective decreases in levels of the thiol antioxidant glutathione in the substantia nigra (SN) early in the course of the disease [1–3]. Although glutathione is not the only antioxidant molecule reported to be altered in PD, the magnitude of glutathione depletion appears to parallel the severity of the disease and is the earliest known indicator of nigral degeneration [4–6]. Along with changes in glutathione levels, iron levels have been reported to be selectively elevated in the Parkinsonian SN at later stages of the disorder. Under normal circumstances, cellular iron homeostasis is primarily maintained by coordinated regulation of levels of proteins by the actions of iron regulatory proteins (IRPs, [7]). When the cellular iron pool is low, IRPs bind to iron-regulatory elements (IREs) present in the 5' untranslated region of ferritin mRNA and the 3' untranslated region of transferrin receptor (TfR1) mRNA. This binding prevents initiation of translation of the ferritin mRNA resulting in decreased iron storage capacity via decreased ferritin protein levels. Concurrently, IRP binding inhibits degradation of TfR1 mRNA, increasing iron transport into the cell by raising TfR1 protein levels. Conversely, high intracellular iron levels normally promote dissociation of IRPs from IREs resulting in up-regulation of ferritin and down-regulation of TfR1 resulting in decreases in the labile iron pool (LIP). Glutathione depletion in dopaminergic cells *in vitro* results in elevations in nitric oxide (NO) levels [8,9]. Drapier and colleagues have suggested that NO and its nitrosonium derivatives can target the electron-rich Fe-S center of IRP1 to produce S-nitroso-IRP1 which constitutively binds IREs as an apoprotein [10]. When

NO levels are sustained as they are following chronic dopaminergic glutathione depletion, this could result in aberrantly persistent IRP1 binding and dysregulation of iron homeostasis. The IRE binding activity of IRP1 has also been shown to be induced by H_2O_2 [11–15] which we have previously demonstrated to also be increased following dopaminergic glutathione depletion *in vitro* [8,9]. Oxidative stress induced by glutathione depletion could also result in induction of hypoxia inducible factor (HIF) that in turn can result in increased TfR1 levels and subsequent iron intake via increased TfR1 transcription [16–18]. Increases in either reactive nitrogen species (RNS) or reactive oxygen species (ROS) as a consequence of glutathione depletion in susceptible dopaminergic neurons could therefore theoretically result in alterations in ferritin and/or TfR1 levels via effects on either the IRP/IRE or HIF pathways in turn impacting on cellular iron homeostasis. Studies were conducted in order to explore whether dopaminergic glutathione depletion *in vitro* results in alterations in cellular iron levels and the possible mechanisms involved.

Materials and methods

Reagents

Chemicals used for all assays were obtained from Sigma (St Louis, MO, USA) unless otherwise noted.

Cell culture and treatments

Dopaminergic N27 cells were grown on poly-L-lysine coated plates (Greiner, Monroe, NC) in medium containing RPMI-1640 medium (Cellgro, Manassas, VA), 10% fetal bovine serum (Clontech, Mountain View, CA), and 10 ml/L of antibiotic antimycotic solution (Cellgro, Manassas, VA). Glutathione was depleted by treatment of cells with buthionine sulfoxamine (BSO) at a concentration of 0–20 μ M for 0–24 to 36 h; previous studies have demonstrated that 20 μ M BSO results in a maximal 50% reduction in cellular glutathione [9]. Co-treatments included catalase (1 mg/ml), cyclohexamide (CHX, 10 μ M), hygromycin (0.1 mg/ml) or G418 (0.2 mg/ml). Cells were treated with 3,4-dihydroxybenzoate (DHB, 200 μ M) as a positive control for HIF activation. After each treatment cells were washed with Hank's buffered salt solution prior to further analysis.

LIP measurements

The fluorescent probe calcein, which is quenched in the presence of iron (Fe^{3+}), was used to measure the labile iron pool [19]. Cells were loaded with .25 mM calcein AM for 30 min at room temperature, washed 3 $\times$ with PBS to remove free dye, and counted. Calcein-loaded cells were then inoculated onto 96-well Optiplates (Perkin-Elmer Life Sciences, Boston, MA) at a density of 50,000 cells per well in 100 μ l of PBS. Immediately before fluorescent measurements, SIH (cell permeable iron chelator, kindly provided by Dr P Ponka, Canada) was diluted in PBS and 100 μ l was added to the plates to give a final concentration of 100 μ M for SIH. Triplicate wells were used for each condition. The plate was then read for 5-min intervals over 30 min on a Molecular Devices fluorescent plate reader (488-nm excitation and 535-nm emission). Fluorescent measurement at each time point for each treatment condition was averaged for the triplicate wells and graphed as a change in relative fluorescent units compared to untreated control cells.

2', 7'-Dichlorofluorescein diacetate (DCF) and 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM) measurements

ROS and nitrosonium (NO^+) levels were measured using the fluorescent probes 2'-7'-dichlorofluorescein diacetate (DCF) and 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM), respectively (both from Molecular Probes, Eugene, OR). DCF-diacetate

or DAF-FM diacetate were loaded directly into the media at 5 mM for 30 min. After loading, the cells were washed with PBS, counted and loaded into 96 well-plate at 50,000 cells per well. The fluorescence was then measured on a Molecular Devices fluorescent plate reader at excitation/emission wavelengths of 488/525 nm for DCF and 495/515 nm for DAF-FM, respectively.

IRP binding assays

Cytoplasmic IRP binding activities were assessed via an RNA gel shift assay using the I12CAT plasmid (gift of Dr. MW Hentze EMBL, Heidelberg, Germany) which contains the IRE sequence of the human ferritin heavy chain under the control of T7 phage promotor. The plasmid is used to prepare IRE RNA probe for the assay via *in vitro* transcription and ^{32}P labeling using an RNA gel shift kit from Fermentas Inc. according to the manufacturer's instructions (Glen Burnie, MD). Cellular homogenates (10 μg) were incubated with a molar excess of the ^{32}P -labeled IRE probe as previously described [20]. IRE-IRP1 complexes were then resolved on 6% non-denaturing acrylamide/bisacrylamide gels and quantified via autoradiography. Betamercaptoethanol (β -ME) was added to aliquots of each sample as a control for loading.

TfR1 real time quantitative PCR

Total RNA was extracted from N27 dopaminergic cells using Trizol (Invitrogen, Carlsbad, CA) and 2 μg was used to generate first strand cDNA by using the SuperScript II reverse transcriptase kit (Invitrogen). Approximately 25 ng of cDNA was subject to Real Time PCR analysis using the SYBR Green PCR Master Mix on ABI prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA). Primers for ferritin H and L chain, TfR1, GAPDH were designed based on nucleotide sequences in Genbank. Values were calculated as relative changes in gene expression in TfR1 normalized to GAPDH via the comparative C_t or $2^{-(\Delta\Delta C_t)}$ method [21]. Here, $\Delta\Delta C_t = \Delta C_{t, \text{sample}} - \Delta C_{t, \text{reference}}$ (C_t is the point on curve at which the point of fluorescence begins to exponentially increase; $\Delta C_{t, \text{sample}}$ is the difference between C_t of TfR1 and C_t of GAPDH at 6, 12, or 24 hrs; $\Delta C_{t, \text{reference}}$ is the difference between C_t of TfR1 and C_t of GAPDH at 0 hrs).

Western blot analyses

Cells were homogenized in lysis buffer (0.15 M NaCl, 5 mM EDTA, pH 8, 1% Triton X100, 10 mM Tris-Cl, pH 7.4, DTT 50 mM) and centrifuged at $10,000\times g$. Protein concentration was determined in the supernatant using Bradford reagent (Bio-Rad, Hercules, CA), 100 μg of protein per sample was mixed with sample buffer (100 mM Tris, pH 6.8, 2% SDS, 5% β -ME, 15% glycerol), boiled and subjected to SDS-PAGE on a 4–15% gel. After transfer to PVDF membranes, the blots were probed with antibodies against TfR1 (Zymed, San Francisco, CA, 1:1000), ferritin (Alpha Diagnostics International, San Antonio, TX, 1:2000), HIF-1 α (Novus, Littleton, CO, 1:1000) or actin (Sigma, St Louis, MO, 1:1000). The proteins were then detected using appropriate secondary antibodies via ECL reagent (Amersham Biosciences, Piscataway, NJ).

Statistical analyses

Values for all data were expressed as means $\pm$ SEM and significance assessed by one way analysis of variance (ANOVA) followed by Newman-Kuels test (Instat, San Diego, CA) or by student's t-test. Differences were considered significant at $P < 0.05$. Data was collected from at least three cultures per condition in at least three separate experiments for all analyses.

Results

Reduction of GSH in N27 cells in vitro results in increases in the LIP

We first assessed the impact of glutathione depletion on the labile iron pool (LIP) following treatment with BSO, an inhibitor of γ -glutamyl-cysteine ligase (GCL), the rate-limiting factor in *de novo* glutathione synthesis. BSO concentrations in the range of 20 μ M have previously been demonstrated to result in a ~50% decrease in levels of total cellular glutathione in these cells in a 24 h period with no significant cell death [9]. Treatment of cells with 20 μ M BSO for 24 h was also found to result in a significant increase in the LIP (Fig. 1).

Glutathione reduction does not impact on IRP1 signaling

As previously demonstrated by our laboratory [8], glutathione reduction in these cells *in vitro* results in increased levels of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Fig. 2). These increases were found to be concentration-dependent following a 24 h treatment period (Figs. 2A and B). Both ROS and RNS have been reported to bind IRP1 and result in constitutively increased IRP1 binding that may result in reduced ferritin, increased TfR1 protein levels and increased cellular LIP [10–12]. However, no change in IRP1 binding was noted following treatment of cells with increasing dosages of BSO (Fig. 2C). In addition, although a significant increase in TfR1 protein levels was noted with increased BSO treatment time (Fig. 4, top panel), there was no change in ferritin levels (data not shown), inconsistent with constitutive IRP binding to these transcripts, suggesting the TfR1 (and subsequent LIP) increase is not IRP-dependent.

Glutathione reduction does not result in changes in TfR1 mRNA

It is possible that the observed increase in TfR1 protein levels is due to oxidatively-induced HIF-1 α activation following glutathione depletion resulting in increased TfR1 transcription. However, no increases in TfR1 RNA levels were noted following 6–24 h of BSO treatment although induction of HIF-1 α via addition of the prolyl hydroxylase inhibitor DHB to the cells did result in increased levels (Fig. 3). In addition, HIF-1 α protein levels were found to remain unchanged following BSO treatment for up to 24 h compared to untreated cells (data not shown). These data taken together suggest that the increase in TfR1 protein is not HIF-dependent.

Glutathione-induced TfR1 protein and LIP increases are both H₂O₂ and protein synthesis-dependent

A recent paper by Andriopoulos et al [22] demonstrated that TfR1 protein levels can be oxidatively induced in inflammatory cells via H₂O₂ release by a protein synthesis-dependent mechanism independent of IRPs. To test whether induction of TfR1 protein levels following BSO treatment in our cells was also protein-synthesis dependent, we assessed TfR1 protein levels in the absence and presence of the protein synthesis inhibitors cyclohexamide (CHX), G418, or hygromycin via western blot analyses (Fig. 4A). BSO-mediated induction of TfR1 protein levels was found to be prevented in the presence of all three of these agents. In order to assess the impact of increased H₂O₂ as a consequence of glutathione depletion as previously observed in these cells (Hsu et al., 2005) on TfR1 protein levels, we examined TfR1 protein levels in the absence and presence of catalase (Fig. 4A). As in the case of the protein synthesis inhibitors, catalase (but not heat-inactivated catalase) also prevented the BSO-mediated TfR1 protein increase. Additionally, this concentration of catalase was able to effectively decrease ROS levels in the presence and absence of BSO (Fig. 4B). Next we assessed the impact of both protein synthesis inhibition via G418 addition and H₂O₂ scavenging via catalase addition on the BSO-mediated increase in LIP (Figs. 5A and B, respectively). Both agents prevented the BSO-induced increase of the LIP. Induction of both TfR1 and LIP levels following glutathione

depletion in our cells is therefore dependent on both H₂O₂ and protein synthesis. Although the concentrations of protein synthesis inhibitors used induced 20–30% cell death (as measured by MTT assay, data not shown), equal amounts of protein and cell number were used for immunoblotting of TfR1 and for LIP measurements, respectively.

Discussion

Glutathione depletion is one of the earliest observable events in the substantia nigra, the brain region primarily affected in PD, but whether or not it contributes to downstream events involved in progressive neurodegeneration including iron increase and via what mechanism(s) has been to date largely unexplored. Using a rat midbrain dopaminergic cell line derived from the same cells lost in the human disease as a model, we provide evidence demonstrating that glutathione depletion in these dopaminergic cells at levels observed in the human condition (~50%) results in an increase in the cellular LIP. This is accompanied by increases in both cellular ROS and RNS levels. Mechanistically, the increase in LIP does not appear to involve induction of either IRP or HIF signaling but is H₂O₂-dependent and coincides with increased translation of the iron uptake protein TfR1. Recent work by Andriopoulos et al. in inflammatory cells has demonstrated that sustained H₂O₂ release can result in up-regulation of TfR1 and cellular iron accumulation independent of either IRP or HIF pathways or increased TfR1 protein stabilization but rather linked to increased TfR1 protein synthesis [22].

In neurons, previous work has suggested that cell death as a consequence of the Parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPTP) in both dopaminergic SY5Y cells and primary cerebellar granule neurons is attributable to TfR1-dependent iron uptake [23]. In non-dopaminergic cerebellar granule cells, TfR1 was found to be activated via oxidatively-induced increases in IRP1 binding [24]. Our data, however, suggests that neither IRP nor HIF induction are not involved in the glutathione-dependent LIP increase observed in our cellular midbrain dopaminergic model system. Rather, our data suggests that sustained, non-toxic H₂O₂ increases as a consequence of glutathione depletion can stimulate TfR-mediated iron increases via a translational mechanism. H₂O₂ has previously been shown to stimulate translation in several systems dependent on the levels of the oxidant and cell sensitivity [25–28]. Our data provides novel evidence connecting glutathione depletion to increases in cellular iron, two important hallmarks of PD, via a here-to-for undescribed mechanism in neurons involving H₂O₂ induction of TfR1 translation. This provides a possible pathophysiological link between dysregulation of iron metabolism in PD and glutathione loss in susceptible dopaminergic midbrain neurons.

Acknowledgments

These studies were funded via NIH R01NS041264 (JKA) and fellowships from the National Parkinson's (DK) and the Larry L. Hillblom (DL) Foundations.

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Fig. 1. Glutathione depletion in dopaminergic N27 cells results in an increase in the LIP. LIP measurements based on SIH dequenching of fluorescent calcein quenching by endogenous iron in BSO-treated (BSO, 20 μ M, 24 h) versus untreated controls (CON). Values are reported as average fluorescent units (AFU) over time (min). * $p < 0.01$, BSO-treated compared to control.

Fig. 2.

Glutathione depletion via BSO results in a concentration-dependent increase in both ROS and RNS levels but no change in IRP binding. (A) ROS levels as assessed by DCF fluorescence following addition of 0, 10, and 20 μ M BSO for 24 h. Values are reported as relative fluorescence units (RFU); * p <0.01 versus time zero. (B) RNS levels as assessed by DAF fluorescence following addition of 0, 10, and 20 μ M BSO for 24 h. Values are reported as relative fluorescence units (RFU); * p <0.01 versus time zero. (C) Cytoplasmic IRP binding as assessed via a RNA gel shift assay in cell homogenates treated with increasing dosages (0, 5, 10, 15, 20 μ M) BSO. β -ME addition was used as loading control.

Fig. 3.

TfR1 message levels are increased by addition of the HIF-inducing agent DHB but not glutathione depletion via BSO. RT-PCR message levels of TfR1 in the presence of 20 μ M BSO or 200 μ M DHB for 6, 12, or 24 h are reported as $2^{-\Delta\Delta C_t}$; * $p < 0.01$ compared to untreated controls (c).

Fig. 4.

(A) BSO-mediated increases in TfR1 protein levels are prevented by both inhibition of protein synthesis and H_2O_2 scavenging. Upper panel: representative western blot analysis of TfR1 protein levels in the presence of 0 (control, C) or 20 μM BSO (B) +/- 10 μM CHX, 0.2 mg/ml G418, 0.1 mg/ml hygromycin, 1 mg/ml catalase, or heat-inactivated catalase (h.i. catalase) for a 24 hr period; actin was used as a loading control. Blots shown are representative of at least three independent experiments. Lower panel: densitometric quantification of band densities (control, CON versus BSO); reported as percent of control normalized to actin. (B) ROS levels as assessed by DCF fluorescence following addition of 20 μM BSO in the presence and absence

of catalase for 24 h. Values are reported as relative fluorescence units (RFU); $*p<0.05$ and $^{\wedge}p<0.0001$ versus CON.

Fig. 5.

BSO-mediated LIP increase is prevented by either inhibition of protein synthesis or H_2O_2 scavenging. (A) LIP measurements in BSO-treated (BSO, 20 μ M, 24 h) versus untreated controls (CON) in the absence and presence of G418 (G418, 0.2 mg/ml). Values are reported as average fluorescent units (AFU) over time after SIH addition (min). * p <0.01 versus control. (B) LIP measurements of BSO-treated (BSO, 20 μ M, 24 h) versus untreated controls (CON) in the absence and presence of catalase (CATA, 1 mg/ml). Values are reported as average fluorescent units (AFU) over time after SIH addition (min). * p <0.01 versus control.