Original Contribution

PROGRESSIVE IRON ACCUMULATION INDUCES A BIPHASIC CHANGE IN THE GLUTATHIONE CONTENT OF NEUROBLASTOMA CELLS

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Abstract—Glutathione (GSH) constitutes the single most important antioxidant in neurons, whereas iron causes oxidative stress that leads to cell damage and death. Although GSH and iron produce opposite effects on redox cell status, no mechanistic relationships between iron and GSH metabolism are known. In this work, we evaluated in SH-SY5Y neuroblastoma cells the effects of iron accumulation on intracellular GSH metabolism. After 2 d exposure to increasing concentrations of iron, cells underwent concentration-dependent iron accumulation and a biphasic change in intracellular GSH levels. Increasing iron from 1 to 5 μM resulted in a marked increase in intracellular oxidative stress and increased GSH levels. Increased GSH levels were due to increased synthesis. Further increases in iron concentration led to significant reduction in both reduced (GSH) and total (GSH + (2 × GSSG)) glutathione. Cell exposure to high iron concentrations (20–80 μM) was associated with a marked decrease in the GSH/GSSG molar ratio and the GSH half-cell reduction potential. Moreover, increasing iron from 40 to 80 μM resulted in loss of cell viability. Iron loading did not change GSH reductase activity but induced significant increases in GSH peroxidase and GSH transferase activities. The changes in GSH homeostasis reported here recapitulate several of those observed in Parkinson's disease substantia nigra. These results support a model by which progressive iron accumulation leads to a progressive decrease in GSH content and cell reduction potential, which finally results in impaired cell integrity.

Keywords—SH-SY5Y cells, Oxidative stress, Redox potential, Viability, Parkinson's Disease, Free radicals

INTRODUCTION

Brain cells have a highly active oxidative metabolism with elevated levels of ATP consumption, yet they contain only low to moderate superoxide dismutase, catalase, nd glutathione peroxidase (GPx) activities [1,2]. Thus, their antioxidant defenses rely mainly on cellular glutathione (GSH) levels [3–5]. GSH reacts nonenzymatically with free radicals, serves as electron donor for GPx-catalyzed reduction of peroxides, and interacts with sulfhydryl groups of proteins to keep them in the reduced state. These functions are fundamental to maintain the intracellular thiol redox potential [6] and involve the oxidation of GSH into its disulfide form, GSSG. GSH is regenerated from GSSG in a reaction catalyzed by GSH reductase (GR), which transfers electrons from NADPH to GSSG. In addition, GSH is nonoxidatively consumed via generation of glutathione *S*-conjugates by glutathione *S*-transferase (GT) [7]. GSH also reacts with nitric oxide and peroxynitrite [8].

Brain antioxidant defenses function properly during most of human life. However, a number of neurodegenerative processes, which involve reactive oxygen (ROS) and nitrogen species, become evident with age [9]. In particular, changes in GSH levels occur in Alzheimer's disease (AD) and Parkinson's disease (PD). Elevated GSH levels in hippocampus and midbrain were reported in AD [10], whereas treatment of NT2 cells with Aβ-amyloid

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peptide decreases GSH and GR activity [11]. Similarly, decreased activity of antioxidant enzymes occur in AD brains [12]. Altered GSH metabolism seems to be an important factor contributing to the pathogenesis of neurodegeneration in PD [13]. Initial studies reported a complete absence of GSH in the substantia nigra of PD patients [14]. Later studies found a 30–40% decrease in GSH concentrations, without a corresponding increase in the levels of GSSG [15]. Interestingly, because nigral GSH levels are decreased to the same extent in presymptomatic PD (incidental Lewy body disease), this decrease seems to be a primary event in the pathogenesis of PD [16]. In addition, GSH levels in PD are specifically decreased in substantia nigra pars compacta (SNpc), without a concomitant increase in the levels of GSSG [17]. The decrease in GSH content correlates with the severity of neurodegeneration [18,19]. Thus, substantial evidence points to decreased levels of GSH in PD and, probably, in AD.

Transition metals such as iron and copper are frequently associated with eurodegenerative processes [20–23]. Iron, because of unpaired electrons in its 3d orbital, participates in a myriad of electron transfer processes, including the electron transport chain [24]. Through the Fenton reaction, Fe^{2+} transforms the mildly oxidant hydrogen peroxide into hydroxyl radical ($HO^{\cdot}$), one of the most reactive species in nature [21]. The Fenton reaction follows mass action law, so $HO^{\cdot}$ production is proportional to reactive Fe^{2+} concentration. There are no known specific mechanisms to detoxify $HO^{\cdot}$, so this species quickly reacts and modifies lipids, proteins, and DNA [21,25].

Iron accumulates with age in redox-sensitive tissues such as substantia nigra [26] and hippocampal neurons [27]. Postmortem studies describe higher levels of iron in normal SNpc than in other brain regions and increased iron accumulation in SNpc of patients with PD [19,26–30]. These findings led to the hypothesis that excess iron is the cause of dopaminergic neuronal death in PD [13]. Similarly, iron has been involved in the etiology of AD [31]. Redox-active iron is found associated with senile plaques and neurofibrillary tangles [22]. Abundant evidence indicates that neurons from Alzheimer brains are subject to a high oxidative load. Postmortem analysis of Alzheimer patient brains revealed activation of heme oxygenase [32] and NADPH oxidase [33], two enzymatic indicators of cellular oxidative stress. The prevalence of oxidative stress in Alzheimer brains led to the proposal that production of free radicals is an early event in the generation of the disease [9,31–33]. Increased iron supply could indeed accelerate neurodegenerative changes. Population studies which indicate that hemochromatosis patients have an earlier onset of Alzheimer's disease than normal individuals [34] support this proposal.

Under the hypothesis that iron accumulation may alter GSH homeostasis and thus impair cell viability, in this work we analyzed the interrelationships between iron and GSH metabolism. To that end, SH-SY5Y neuroblastoma cells were loaded with varied contents of iron and both GSH levels and the activity of enzymes involved in GSH metabolism were studied. The results show that after an initial increase in GSH synthesis, iron accumulation produced decreased levels of GSH and increased levels of GSSG, to a point at which cell viability was impaired. These results indicate that cell GSH levels are a target of iron accumulation.

MATERIALS AND METHODS

Materials

Fetal bovine serum, MEM and F12 culture media, protease inhibitors, buffers, and salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorescin diacetate (DCDHF-DA) were from Molecular Probes (Eugene, OR, USA). ^{55}Fe in the ferric chloride form was obtained from New England Nuclear (Boston, MA, USA). Culture plasticware and Transwell bicameral inserts were from Corning Costar (Cambridge, MA, USA).

Cell culture and iron challenge

Human neuroblastoma SH-SY5Y cells (CRL-2266; American Type Culture Collection, Rockville, MD, USA) were seeded at 1×10^5 cells in 2 cm^2 plastic wells and cultured in a 5% CO_2 incubator in MEM/F12 medium supplemented with 10% fetal bovine serum and 5 mM glutamine. The medium was replaced every 2 d. Under these conditions, doubling time was about 48 h. After 8 d in culture the culture reached a steady-state number of cells. At this time, cells were challenged with iron for the next 2 d. To that end, culture medium was changed to MEM, 10% low-iron FBS (iron-depleted fetal bovine serum; total iron content <0.2 μM) [35], supplemented with 1, 5, 10, 20, 40, or 80 μM Fe^{3+} as the complex $FeCl_3$–sodium nitrilotriacetate (NTA, 1:2.2, mol:mol) [36]. One micromolar Fe was considered the basal condition, because it corresponds to the reported iron concentration of cerebral spinal fluid [21]. This model of iron loading intends to recapitulate neuronal iron accumulation which may occur during long (years) periods of time [37].

^{55}Fe uptake and cell viability

Cells grown for 8 d as described above were changed to low-Fe medium supplemented with 1, 5, 10, 20, 40, or 80 μM Fe^{3+} containing ^{55}Fe–NTA as a tracer. After 2 d,

cells from triplicate wells were harvested, cell extracts were prepared [36], and their ^{55}Fe content was determined. Iron uptake was expressed as picomoles of ^{55}Fe per milligram of cell protein.

Cell viability was quantified by the MTT assay following the manufacturer's instructions. This assay determines the mitochondrial-dependent formation of a colored product [38].

Assessment of oxidative stress

Generation of reactive oxygen species was determined using the membrane-permeable fluorescent dye DCDHF-DA. DCDHF-DA is hydrolyzed inside cells to the non-fluorescent compound 2′,7′-dichlorodihydrofluorescin, which emits fluorescence when oxidized to 2′,7′-dichlorofluorescein (DCF). Thus the fluorescence emitted by DCF directly reflects the overall oxidative status of a cell [39]. Coverslip-grown cells were cultured for 2 d in medium with varied iron concentrations as described above. The medium was made 10 μM in DCDHF-DA and incubation continued for 45 min. The cells were washed three times with saline and DCF fluorescence was determined in a Zeiss Axiovert 200M epifluorescence microscope equipped with temperature and CO_2 controllers. Temperature was maintained at 37°C and CO_2 at 5%. Low-magnification fields from each culture were imaged with an Axiocam HR camera attached to a PC equipped with Axiovision software. Shutters and a neutral density filter were used to minimize photobleaching. Fluorescence intensity from at least 100 cells from the captured images was determined using the Quantity One (Bio-Rad) software. Fluorescence values were expressed as arbitrary fluorescence intensity units.

Determination of GSH and GSSG levels and enzyme activities

GSH and GSSG levels were assayed as described [40,41]. GSH reductase was determined using a commercial kit (359962; Calbiochem). Established methods were used to determine the activity of GSH peroxidase [42] and GSH transferase [43].

Estimation of the GSH/GSSG redox potential

The redox potential for the GSH/GSSG half-cell was determined as described [6], using the equation

$$E_{hc} = -240 - (59.1/2)\log([GSH]^2/[GSSG]), mV,$$

where −240 (in mV) corresponds to the standard reduction potential of the GSH/GSSG couple.

Data analysis

Variables were tested in triplicate, and experiments were repeated at least twice. Variability among experiments was <20%. One-way ANOVA was used to test differences in mean values, and Tukey's post hoc test was used for comparisons (In Stat program from GraphPad Prism). Differences were considered significant if $p < .05$.

RESULTS

Increasing the concentration of iron in the culture medium from 1 to 80 μM resulted in a concentration-dependent increase in the intracellular iron content of SH-SY5Y cells (Fig. 1A). Cell viability remained over 90% up to 40 μM Fe, dropping to 59% ($p < .01$) in cells exposed for 2 d to 80 μM Fe (Fig. 1B). A direct relationship was found between iron load and

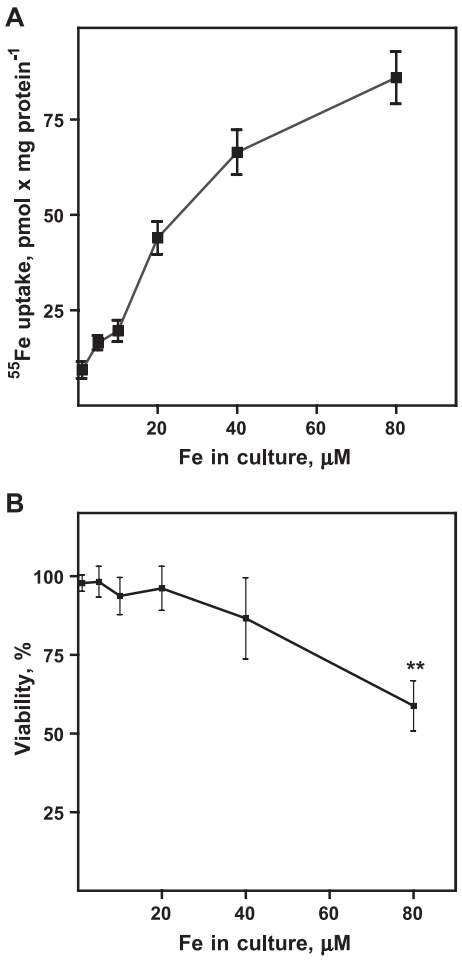

Fig. 1. Progressive iron loading of SH-SY5Y cells. (A) Relationship between extracellular and intracellular iron content. Cells were seeded and grown for 8 d in standard culture medium and then cultured for 2 d in a medium containing 1, 5, 10, 20, 40, or 80 μM Fe with ^{55}Fe added as tracer. The iron content taken up by the cells was expressed as pmol/mg of cell extract protein. Note the continuous increase in cell iron uptake in the 1–80 μM extracellular iron range. (B) Cell viability. Cell viability under the culture conditions described above was determined by the MTT method. Culture for 2 d in 80 μM Fe resulted in a significant decrease in cell viability ($p < 0.01$ compared to the 1 μM value).

DCF fluorescence in the 5 to 40 μM Fe range, with DCF fluorescence distributed evenly throughout the cells (Fig. 2A). High-magnification images revealed occasional zones of higher fluorescence in a thread-like pattern (not shown). Quantification of the oxidative load in these cells revealed that ROS increased markedly in the 1–40 μM Fe range (Fig. 2B). Thus, a direct relationship was found between iron load and ROS production in SH-SY5Y cells.

Cells cultured with these various concentrations of iron revealed a biphasic pattern of change in GSH levels (Fig. 3A). When iron was raised from 1 to 5 μM intracellular GSH levels increased significantly ($p < .01$) from 12.9 to 23.4 nmol/mg protein. However, at extracellular concentrations of iron higher than 5 μM, a progressive decrease in the intracellular GSH concentration, down to 10.8 nmol/mg protein, was observed. GSSG levels were very small, increasing steadily in the 1–80 μM Fe range (Fig. 3A). Total GSH equivalents (GSH + (2 × GSSG)) increased in the 1–5 μM Fe range and decreased significantly ($p < .001$) in the 5–80 μM Fe range (Fig. 3B).

Because of its elevated cellular concentration, GSH is a major contributor to the maintenance of the redox potential in neuronal cells [3,5,44]. Thus, the GSH/GSSG molar ratio and the redox potential of the GSH/GSSG couple are accurate descriptors of cellular redox state [6]. The GSH/GSSG ratio was above 60 in the 1–10 μM Fe range, but decreased significantly in the 20–80 μM Fe range, reaching values of 30.5 and 17.5 at 40 and 80 μM Fe, respectively (Table 1). An increase of 22.4 mV in the redox potential of the GSH/GSSG half-cell was obtained when iron increased from 1 to 80 μM (Table 1).

Recent evidence indicates that GSH synthesis can be modulated through transcriptional activation of the catalytic subunit of glutamate cysteine ligase by oxidative stress [45,46]. Thus, we studied the effect of the GSH synthesis inhibitor L-buthionine sulfoximine (BSO) on the initial increase in GSH seen at the lower iron concentrations. Cells incubated with 75 μM BSO did not differ from control cells in their GSH levels when incubated with 1 or 5 μM Fe, whereas the combination of 75 μM BSO and 80 μM Fe resulted in marked inhibition of GSH synthesis (Fig. 4). The decrease in GSH levels seen at 80 μM Fe can be explained in terms of a failure of 75 μM BSO-treated cells to respond to 80 μM Fe-induced oxidative stress. Culture of cells with 1, 5, or

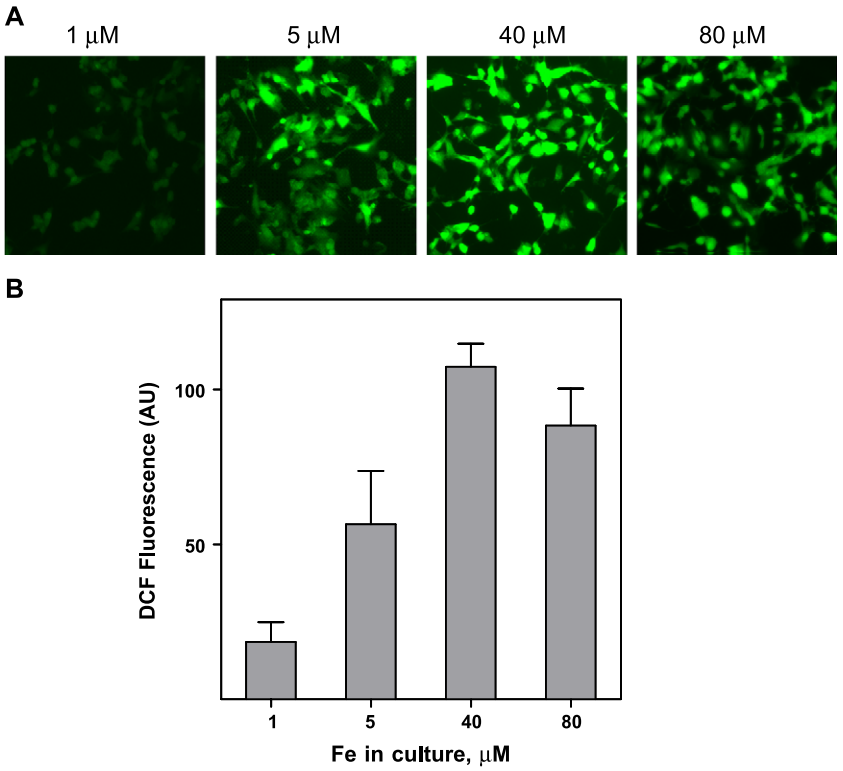

Fig. 2. Iron accumulation induces oxidative stress. SH-SY5Y cells were grown for 8 d in standard culture medium and then cultured for 2 d in medium containing 1, 5, 10, 20, 40, or 80 μM Fe. Cells were loaded with DHCF-DA and DCF fluorescence was determined. (A) Representative images of cells cultured in 1, 5, 40, or 80 μM Fe. (B) Quantification of DCF fluorescence intensity in cells cultured in medium containing different iron concentrations.

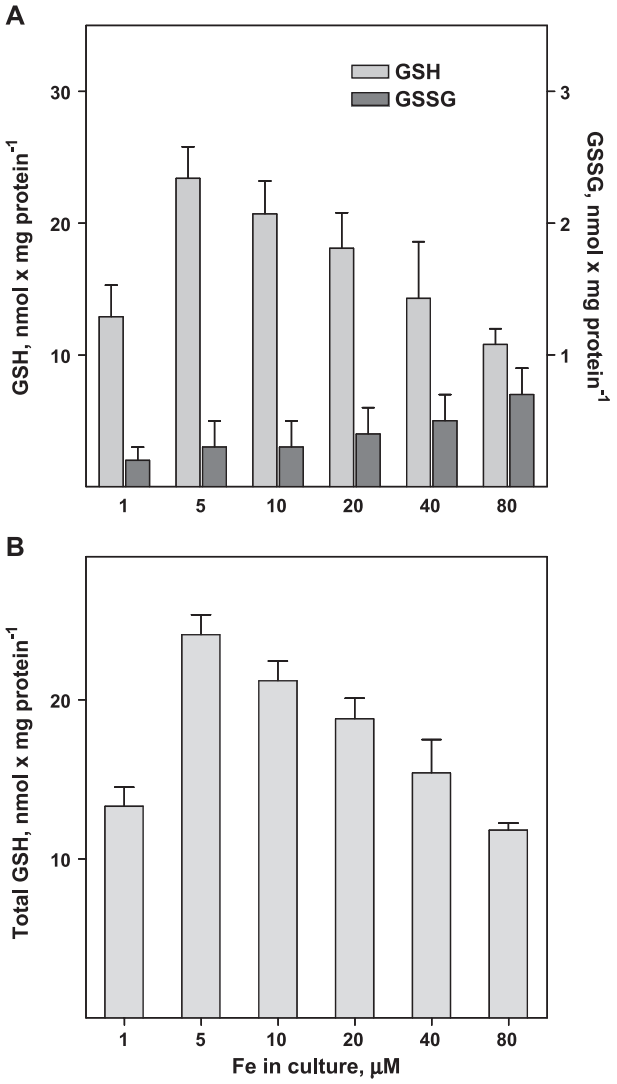

Fig. 3. (A) GSH and GSSG levels in cells subjected to increasing iron loads. Cells were cultured for 8 d in standard culture medium and for 2 d in medium with varied (1–80 μM) iron as described for Fig. 1. Cell extracts were prepared and GSH and GSSG levels were determined. Note the inverse relationship between iron and GSH levels in the range 20–80 μM Fe. (B) Total GSH. Values from (A) were plotted as the total (GSH plus 2 × GSSG) glutathione content as a function of extracellular iron concentration. Values are means ± SD from four independent experiments.

10 μM Fe in the presence of 150 μM BSO resulted in a profound inhibition of GSH synthesis at all iron concentrations tested (Fig. 4). Thus, the capacity to maintain adequate GSH levels was lost in cells supplemented with 150 μM BSO.

Searching for possible causes underlying the iron-induced decrease in GSH content, we assayed GSH-related enzyme activities (Fig. 5). No changes in activity for GR were found with increasing iron concentrations (Fig. 5A), whereas GPx activity increased significantly at 5 μM Fe, remaining high up to 80 μM Fe (Fig. 5B).

Table 1. GSH Half-Cell Redox Potential in Cells Subjected to Different Iron Loads

Fe in the culture (μm)	GSH (nmol protein^{-1})	GSSG mg (nmol protein^{-1})	E_{hc} mg (mV)	ΔE_{hc} (mV)
1	12.93	0.16	−329.0	0
5	23.43	0.35	−334.4	−5.4
10	20.68	0.28	−334.3	−5.3
20	18.14	0.35	−328.0	1.0
40	14.32	0.53	−316.6	12.4
80	10.58	0.63	−306.6	22.4

Values from Fig. 3A (means form four independent experiments) were used to calculate the GSH/GSSG half-cell redox potential (E_{hc}) applying the equation $E_{hc} = -240 - (59.1/2)log([GSH]^2/[GSSG])$. ΔE_{hc} difference in the GSH/GSSG half-cell redox potential compared to the 1 μM condition.

Similarly, GT activity increased significantly in the 5–20 μM Fe range, remaining constant in the 20–80 μM Fe range (Fig. 5C).

DISCUSSION

Iron accumulation and oxidative stress are hallmarks of several neurodegenerative diseases [9]. Nevertheless, the mechanisms by which iron may eventually overcome cellular antioxidant mechanisms are not understood. In this work we used a progressive cell iron-loading model to study the relationship between iron accumulation and GSH metabolism in SH-SY5Y neuroblastoma cells. Our studies indicate that after 2 d exposure to increasing concentrations of extracellular iron neuroblastoma cells

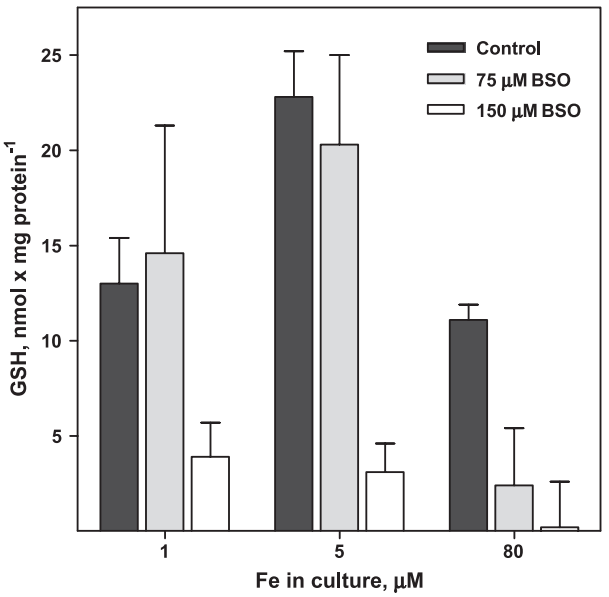

Fig. 4. Effects of BSO on the iron-mediated rise in GSH levels. Cells were cultured for 8 d in standard culture medium and then for 2 d in medium with 1, 5, or 10 μM Fe, without or with the addition of 75 or 150 μM BSO. GSH levels were determined from cell extracts and expressed as a function of Fe in the culture medium.

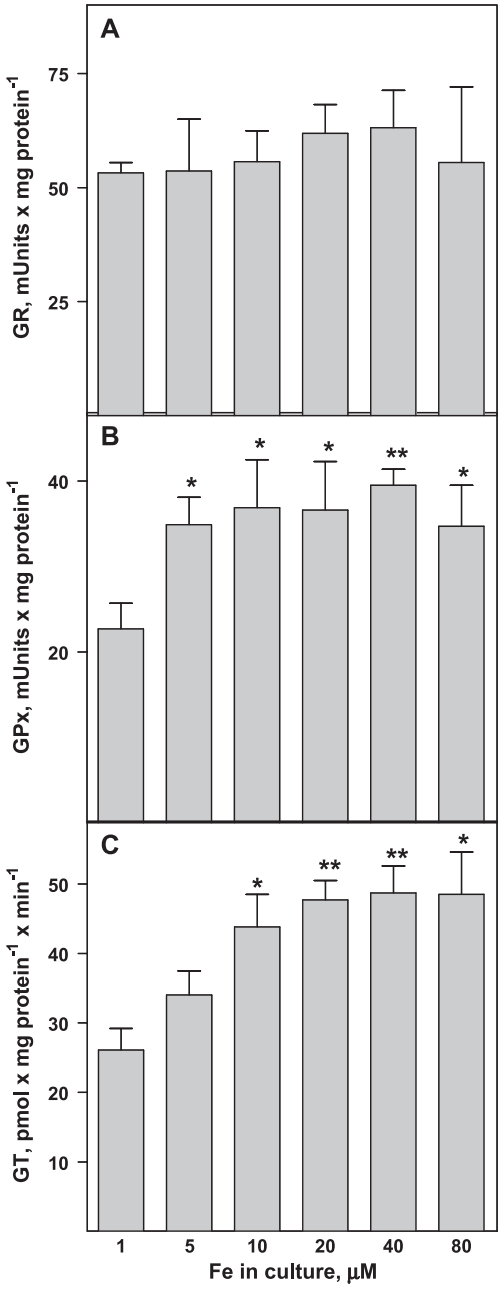

Fig. 5. GSH enzyme activities in cells subjected to increasing iron load. Cells were cultured for 8 d in standard culture medium and then for 2 d in medium with varied (1–80 μM) Fe as described for Fig. 1. Cell extracts were prepared and GR, GPx, and GT activities were determined as described under Materials and Methods. Changes in GR were nonsignificant compared to the 1 μM Fe value. Changes in GPx and GT activities were significantly different (*$p < .05$, **$p < .01$) compared to the 1 μM Fe value.

underwent a concentration-dependent accumulation of iron, accompanied by a biphasic change in the intracellular concentration of GSH and a decrease in the GSH/GSSG ratio. Whereas exposure to 5 μM Fe resulted in a marked increase in GSH levels compared to 1 μM Fe, exposure to higher Fe concentrations, up to 80 μM, decreased GSH levels and increased GSSG levels. Similar results, i.e., decreased GSH and increased GSSH, were observed in the brain of mice subjected to a high-iron diet [47], whereas in PD GSH levels were found specifically decreased in SNpc [15–17].

Increasing extracellular iron concentration from 40 to 80 μM was associated with a marked loss of cell viability. Because iron accumulation increases oxidative stress (Fig. 2 this work; [36]), the initial rise in GSH synthesis may be interpreted as an adaptive response of the cells to iron-mediated oxidative stress. In turn, the subsequent decrease in GSH may correspond to a negative balance between GSH synthesis and Fe-induced GSH consumption. Indeed, inhibition of GSH synthesis by BSO produced increased sensitivity of GSH levels to iron load.

The GSH/GSSG ratio was above 60 in the 1–10 μM Fe range, but decreased to 30.5 and 17.5 at 40 and 80 μM Fe, respectively. Because 80 μM Fe induced considerable loss of cell viability, it is likely that GSH/GSSG ratios below 30 become critical for cell survival. Similarly, changes in the GSH reduction potential to more positive values indicated that iron-induced GSH consumption resulted in a more oxidant cell environment. In opposition to these findings, no increase in GSSG levels was reported in Parkinsonian SNpc [17]. A possible explanation for this difference may reside in the fact that our GSSG determinations were undertaken in freshly harvested neuroblastoma cells, as opposed to postmortem brain tissue. In the latter situation, O_2/Fe-mediated GSH oxidation may overestimate GSSH content. Despite the differences in GSSG content, the marked decrease in GSH found in parkinsonian brains argues for decreased GSH/GSSG ratios, as are reported in this work.

GR activity did not change in the 1–80 μM iron range, suggesting that GR is either already maximally expressed under basal conditions or not inducible by iron. In contrast, GPx and GT activities increased. As observed here, the activity of GR was found unchanged in SNpc of PD, whereas the activity of GT had a tendency to increase [48]. The finding that both GPx and GT activity increased in SH-SY5Y cells exposed to iron agrees with recent reports on gene expression in the MPTP model of PD, in which increases in GPx and GT mRNA were reported [49], but does not agree with earlier findings indicating no change in GT activity in substantia nigra of Parkinson's disease patients [15]. Thus, it is possible that increase in GT may not be a characteristic feature of PD.

The observed increases in GPx and GT may represent defense mechanisms against oxidative stress. We propose that the increase in GSH synthesis, in conjunction with increased GPx and GT activities, is an important element

in the antioxidant response to iron accumulation. In this context, our data support a mechanism by which iron accumulation in SNpc results in decreased GSH content and cell death. However, no increase in iron before GSH depletion was found in substantia nigra of patients with incidental Lewis body disease [16], and initial death of tyrosine hydroxylase-positive cells preceded iron accumulation in monkeys injected with MPTP [50], an indication that iron accumulation could be indeed secondary to dopaminergic cell death.

In summary, we found that iron accumulation in neuroblastoma cells resulted in a biphasic change in the levels of GSH levels and a decrease in cellular redox potential, thus establishing a mechanistic link between iron and GSH homeostasis. Considering that adequate levels of GSH are necessary for neuronal survival, these results point to the relevance of iron accumulation as a primary cause for diminished redox potential leading to neurodegeneration in diseases such as Alzheimer's and Parkinson's disease.

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