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An oxidative stress-mediated positive-feedback iron uptake loop in neuronal cells

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Abstract

Intracellular reactive iron is a source of free radicals and a possible cause of cell damage. In this study, we analyzed the changes in iron homeostasis generated by iron accumulation in neuroblastoma (N2A) cells and hippocampal neurons. Increasing concentrations of iron in the culture medium elicited increasing amounts of intracellular iron and of the reactive iron pool. The cells had both IRP1 and IRP2 activities, being IRP1 activity quantitatively predominant. When iron in the culture medium increased from 1 to 40 μM, IRP2 activity decreased to nil. In contrast, IRP1 activity decreased when iron increased up to 20 μM, and then, unexpectedly, increased. IRP1 activity at iron concentrations above 20 μM was functional as it correlated with increased ^{55}Fe uptake. The increase in IRP1 activity was mediated by oxidative-stress as it was largely abolished by *N*-acetyl-L-cysteine. Culturing cells with iron resulted in proteins and DNA modifications. In summary, iron uptake by N2A cells and hippocampus neurons did not shut off at high iron concentrations in the culture media. As a consequence, iron accumulated and generated oxidative damage. This behavior is probably a consequence of the paradoxical activation of IRP1 at high iron concentrations, a condition that may underlie some processes associated with neuronal degeneration and death.

Keywords: DMT1, hippocampal neurons, iron, IRP, N2A cells, oxidative damage.

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Iron has been shown to be involved in the generation of oxidative damage in several neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotropic lateral sclerosis (reviewed by Sayre *et al*. 2000a). In PD, excess iron accumulates in brain regions sensitive to neurodegeneration such as substantia nigra and globus pallidus (Youdim and Riederer 1993; Griffiths *et al*. 1999). These findings led to the proposition that iron excess is the cause of dopaminergic neuronal death in PD (Double *et al*. 2000). Similarly, iron has been involved in the etiology of AD (Smith *et al*. 1997; Perry *et al*. 2000; Sayre *et al*. 2000a). Redox-active iron is found associated with senile plaques and neurofibrillary tangles (Smith *et al*. 1998; Sayre *et al*. 2000b). Abundant evidence indicates that neurons from Alzheimer's brains are subject to a high oxidative stress load. Post-mortem analysis of AD patient brains revealed, in addition to increased iron levels, an activation of heme oxygenase (Takeda *et al*. 2000) and NADPH oxidase (Shimohama *et al*. 2000), two enzymatic indicators of cellular oxidative stress. The prevalence of oxidative stress in Alzheimer's brains has led to the proposal that the production of free radicals is an early event in the generation of the disease (Perry *et al*. 2000; Sayre *et al*. 2000a). It has recently been reported that hemochromatosis patients have an earlier onset of AD than normal individuals (Connor *et al*. 2001), an indication that increased iron supply in these patients could accelerate neurodegenerative changes.

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Abbreviations used: DCFDA, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HNE, 4-hydroxy-e-nonenal; IRE, iron-responsive element; IRP, iron regulatory protein; MTT, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; N2A, neuroblastoma; NTA, nitrilotriacetate; 8-OhdG, 8-hydroxy-2'-deoxiguanosine; SIH, Salicyladehyde isonicotinoyl hydrazone; Tf, transferrin; TfR, transferrin receptor.

The biology of transition metals is receiving increasing attention due to their ability to generate reactive oxygen species and oxidative damage under physiological conditions. Through the Fenton reaction, Fe^{2+} generates the highly reactive hydroxyl radical (Okada 1996; Symons and Gutteridge 1998). Because of mass-action law, the production of the hydroxyl radical through the Fenton reaction is directly proportional to the concentration of Fe^{2+}. Thus, to avoid oxidative damage, intracellular reactive iron must be kept low.

Iron is essential for life, so despite its potential toxicity, mechanisms have been developed to ensure its adequate supply. In vertebrates, cellular iron levels are post-transcriptionally controlled by the activity of iron regulatory proteins (IRP1 and IRP2), cytosolic proteins that bind to structural elements named iron-responsive elements (IREs; Kühn and Hentze 1992; Kim *et al*. 1995; reviewed by Eisenstein 2000). IREs are found in the untranslated region of the mRNAs of the major proteins that regulate cellular iron homeostasis: the transferrin receptor (TfR), involved in plasma-to-cell iron transport, and the iron-storage protein ferritin. Although no detailed studies exist, expression of the iron transporter DMT1 seems to follow a mechanism similar to that of the TfR, as it has an IRE motif that is functional in IRP binding (Wardrop and Richardson 1999), and its mass increases in conditions of iron-deficiency (Canonne-Hergaux *et al*. 1999; Arredondo *et al*. 2001). The activities of both IRP1 and IRP2 respond to changes in cellular Fe, but through different mechanisms. In iron-replete conditions, IRP1 has a 4S-4Fe cubane that renders the protein active as a cytosolic aconitase but inactive for IRE-binding. Low levels of intracellular Fe induce disassembling of the 4S-4Fe cluster, which causes IRP1 to bind to and stabilize TfR mRNA. Furthermore, IRP1 binds to ferritin mRNA, thus diminishing its translation (Kühn and Hentze 1992; Kim *et al*. 1995; reviewed in Eisenstein 2000). In contrast to IRP1, IRP2 is always active to bind to IREs, but its IRE-binding activity is down-regulated through iron-induced oxidative damage, followed by ubiquitination and proteasome degradation (Iwai *et al*. 1998). Effectors such as nitric oxide (Phillips *et al.* 1996), hydrogen peroxide (Martins *et al*. 1995), hypoxia (Hanson *et al*. 1999), and phosphorylation (Schalinske and Eisenstein 1996) also regulate IRPs. IRP1 is activated by extracellular, but not intracellular, H_2O_2 in CHO and B6 fibroblasts (Martins *et al*. 1995; Pantopoulos *et al*. 1997). The activation of IRP1 by H_2O_2 results in the increased expression of TfRs and increased cell iron uptake (Caltagirone *et al*. 2001), a paradoxical response that could result in an additional iron-mediated oxidative challenge through the Fenton reaction.

Once gained, cells tend to keep their iron tenaciously, cell division being one of the few occasions in which cell iron levels are effectively reduced. Because of their post-mitotic condition, neurons cannot reduce intracellular iron through cell division, so they depend very strictly on the mechanisms of cellular iron homeostasis to manage the risk of iron-mediated oxidative damage. Neurons have an active IRE/IRP system (Hu and Connor 1996; Leibold *et al*. 2001) and express ferritin, TfR and the membrane Fe^{2+} transporter DMT1 (Connor *et al*. 1992; Roth *et al*. 2000; reviewed by Qian and Shen 2001). The localization of IRP2 to senile plaques and neurofibrillary tangles in neurons from Alzheimer's patients, an indication of abnormal iron homeostasis in these cells, has been reported (Smith *et al*. 1998).

Many of the specific features of neuronal iron homeostasis are scarcely known. For example, a better understanding of neuronal IRE/IRP system and the mechanisms by which neurons regulate intracellular iron levels should give important clues about the relevance of iron in the induction of oxidative damage. In this study, we characterized iron homeostasis in neuroblastoma cells and hippocampal neurons, to investigate the ability of these cells to control the levels of reactive iron, and to avoid iron-induced oxidative damage. We found that despite having an active IRE/IRP system, the cells accumulated iron as a function of time and availability. This behavior was most probably a consequence of the oxidative stress-mediated activation of IRP1 at high iron concentrations. Despite increased iron content, neuronal cells managed to stay viable and control the degree of oxidative damage to proteins and DNA, probably through the activation powerful antioxidant mechanisms.

Materials and methods

Materials

Fetal bovine serum, transferrin (Tf), protease inhibitors, culture media, buffers and salts were purchased from Sigma Chemical Co. (St Louis, MO, USA). Calcein-AM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (DCFDA) were from Molecular Probes (Eugene, OR, USA). Salicylaldehyde isonicotinoyl hydrazone (SIH) was the gift of Dr Prem Ponka (Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montreal, Canada). A chicken antibody against a peptide from the protein domain exclusive to IRP2 (Guo *et al*. 1995) was the gift of Dr E. Leibold (Eccles Program in Human Molecular Biology and Genetics, University of Utah, Salt Lake City, UT, USA). Monoclonal antibodies HNEJ-2 and N45.1 were the gift of Dr Shinya Toyokuni (Department of Pathology and Biology Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan). ^{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

Mouse neuroblastoma (N2A) cells (CCL-131, American Type Culture Collection Rockville, MD, USA), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum and 0.2 mM dibutyryl cAMP as inductor of neuronal differentiation (Chen *et al*. 1983). The culture medium was

changed every 2 days. After 6 days in culture, the cells began to detach from the dish, so no experiments were run beyond that time. Hippocampal neurons were prepared from E18.5 rat embryos (Banker and Cowan 1977). Neurons were plated over poly-L-lysine coated coverslips at 15 000 cells/cm^2. Cultures were maintained in 10% bovine serum until 3 h after plating, when the culture medium was replaced with medium containing the N_2 supplement (Gibco-BRL, Gaithersburg, MD, USA; Bottenstein and Sato 1979). The N2 medium was replaced every 48 h.

When challenged with varied concentrations of iron, cells were trypsinized and grown for 3 days in DMEM, 10% fetal bovine serum (FBS), followed by incubation for 2 days in DMEM, 10% low-iron FBS (iron-depleted fetal bovine serum; total iron content < 0.5 μmolar, Arredondo *et al*. 1997) supplemented with either 1, 10, 20, 40 or 80 μM Fe^{3+} as the complex $FeCl_3$–sodium nitrilotriacetate (NTA, 1 : 2, mol:mol). In long-term iron uptake experiments, the culture media contained $^{55}FeCl_3$–NTA.

Cell extracts and band-shift assay

Cell extracts were prepared by treating cells with lysis buffer (50 μL per 1×10^6 cells of 10 mM HEPES, pH 7.4, 3 mM $MgCl_3$, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 0.5 μg/mL aprotinin, 0.7 μg/mL pepstatin A, 5% glycerol, 1 mM dithiothreitol, 0.5% Triton X-100). The mixture was incubated for 15 min on ice, sedimented for 10 min at 10 000 *g* and stored in aliquots at − 70°C.

IRP activity was determined by an RNA band-shift assay performed as described earlier (Leibold and Munro 1988; Arredondo *et al*. 1997). β-Mercaptoethanol was used at 2% (v/v) when noted. When 'super-shift' assays were performed, incubation with antiserum that recognizes the unique 73 amino acid insert of IRP2 for 30 min before electrophoresis was used (Guo *et al*. 1995). Radioactivity detection was done with a Phosphoimager device (Bio-Rad, Hercules, CA, USA). The quantification of IRP1 activity was done obtaining the ratio between the activity in the absence of β-mercaptoethanol and the activity in the presence of β-mercaptoethanol. The activity of the individual bands was determined using Quantity One software (Bio-Rad).

^{55}Fe uptake

Cells seeded at 1×10^5 cells in 2-cm^2 plastic wells were allowed to grow for 2 days in DMEM−10% FBS. The medium was then changed to low Fe medium supplemented with 10 μM $^{55}Fe^{3+}$ as the complex $^{55}FeCl_3$-NTA. Cells from triplicate wells were harvested and ^{55}Fe content was measured for the next 4 days. For studies designed to measure Tf-bound and non-Tf-bound iron uptake, triplicates were incubated for varied times (5–60 min) in MOPS–saline buffer [50 mM 3-(*N*-morpholino) propanesulfonic acid-Na, 94 mM NaCl, 7.4 mM KCl, 0.74 mM $MgCl_2$ and 1.5 mM $CaCl_2$, pH 7.0] with either 5 μM ^{55}Fe-labeled diferric Tf (Tf-bound iron uptake), or with 10 μM of the ^{55}Fe–NTA complex (non-Tf-bound iron uptake).

Measurement of the reactive iron pool

The intracellular labile or reactive iron pool of N2A cells was determined as described (Epsztejn *et al*. 1997; Núñez *et al*. 2001). Briefly, cells were grown on glass coverslips for 2 days in DMEM, 10% FBS followed by incubation for another 3 days in low-iron medium supplemented with 5 μM, 20 μM, 50 μM or 100 μM Fe^{3+} as the complex $^{55}FeCl_3$–NTA. Calcein–AM (0.5 μM, Molecular Probes) was then loaded into the cells for 5 min at 37°C. After washing non-internalized calcein, the cells were transferred to a cuvette containing 3 mL of MOPS–saline pH 7.4 and 5 μL of anticalcein antibody (the kind gift of Dr Z. I. Cabantchik). After determination of the basal calcein fluorescence (excitation 488 nm, emission 517 nm), the fluorescence of the calcein–Fe complex was de-quenched by the addition of 100 μM SIH. The increase in fluorescence thus obtained was directly proportional to the iron labile pool.

Determination of oxidative damage

Neuroblastoma cells grown on glass coverslips were incubated for 2 days with varied amounts of iron in the culture media as described above. Evaluating 4-hydroxy-2-nonenal (HNE)-modified proteins assessed oxidative damage to proteins. HNE is the α,β-unsaturated aldehyde product of the peroxidation of ω6-unsaturated fatty acids that form adducts with amino acid residues such as histidine, lysine, and cysteine. HNE was detected in western blots with monoclonal antibody HNEJ-2, which recognizes HNE–histidyl adducts (Toyokuni *et al*. 1995; Núñez *et al*. 2001). Oxidative damage to DNA was assessed with monoclonal antibody N45.1, which recognizes 8-hydroxy-2′-deoxiguanosine (8-OHdG), a highly specific product of DNA damage (Toyokuni *et al*. 1997). The antibody was developed with the peroxidase-based Vectastin ABC kit (Vector Laboratories, Burlingame, CA, USA). No peroxidase reaction product was observed if the primary antibodies were omitted or replaced by non-immune serum.

Cell viability was determined with DCFDA following manufacturer's instructions. DCFDA is a non-fluorescent molecule that transforms into the fluorescent compound DCF upon hydrolysis. In viable cells, DCF fluorescence reflects ROS levels, while in non-viable cells DCF fluorescence is lost (Provinciali *et al*. 1992). Cell viability was also quantified by the MTT assay following the manufacturer's instructions. This assay determines the mitochondrial-dependent formation of a colored product (Mossman 1986).

Data analysis

Variables were tested in triplicate, and experiments were repeated at least twice. Variability between experiments was <15%. One-way ANOVA was used to test for differences in means, and a *post-hoc t*-test was used for comparisons. Differences were considered significant if $p < 0.05$.

Results

Fe uptake by neuroblastoma cells

We examined the characteristics of iron uptake by neuroblastoma cells cultured for several days in a medium containing 10 μM ^{55}Fe (Fig. 1a). The cells acquired increasing amounts of iron with time under culture conditions. No shut-off of iron uptake was observed after 4 days of culture, although a decrease in the rate of ^{55}Fe uptake was evident. This behavior was similar for control cells and for cells differentiated to a neuronal phenotype with 0.2 mM dibutyryl

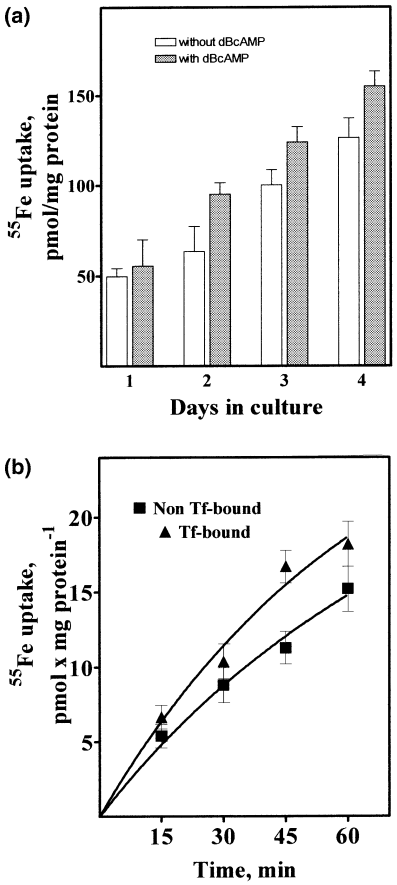

Fig. 1 Fe uptake by neuroblastoma cells. (a) Long-term iron uptake by neuroblastoma cells. Cells were cultured in the presence of 10 μM ^{55}Fe. At different days of culture, cells were analyzed for cell-associated ^{55}Fe. No arrest in ^{55}Fe uptake was observed during the period of the experiment in both control cells or cells differentiated to a neuronal phenotype with dibutyryl cAMP. (b) Tf-bound (▲) and non-Tf-bound (■) ^{55}Fe uptake. ^{55}Fe uptake either from 5 μM diferric ^{55}Fe Tf, or from 10 μM ^{55}Fe-NTA is shown. Values represent the mean ± SD of two independent experiments done in triplicate.

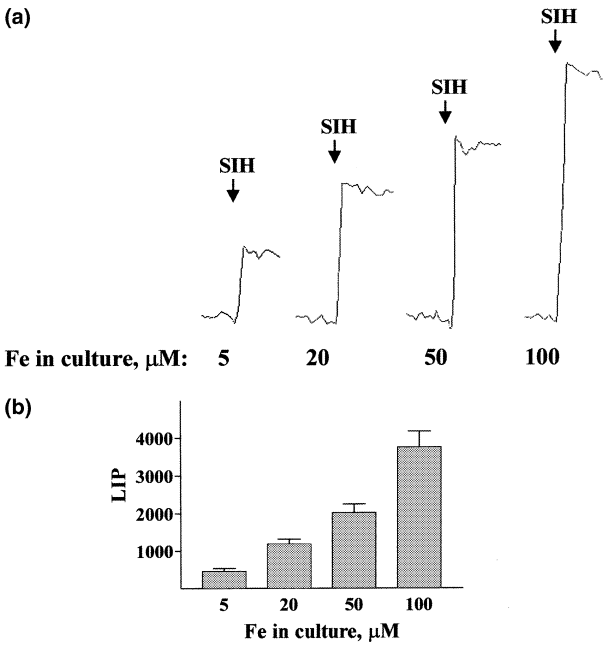

Fig. 2 Reactive iron pool in neuroblastoma cells. Cells grown on coverslips were cultured for 4 days in regular culture media and then cultured for 24 h in media containing 5, 20, 50, or 100 μM Fe. The reactive iron pool was then measured by SIH de-quenching of calcein fluorescence. SIH is a membrane-permeant Fe chelator that takes Fe from the calcein–Fe chelate, thus increasing calcein fluorescence. Therefore, the level of the cellular labile Fe pool is directly proportional to the increase in SIH-induced calcein fluorescence. Representative traces of intracellular calcein fluorescence (a) and means ± SD from four independent determinations (b).

cAMP. Neuroblastoma cells acquired equally well iron from Tf (triangles, Fig. 1b) or from a Fe–NTA complex in the absence of Tf (squares, Fig. 1b). Iron uptake from Tf indicated the presence of TfRs undergoing an active endocytic cycle, while iron uptake from non-Tf-bound iron denoted the presence of a membrane transporter, most probably DMT1(Roth *et al*. 2000).

We then investigated how changes in culture media iron were reflected in the reactive or labile iron pool. Increasing concentrations of iron in the culture media increased the calcein-sensitive iron pool (Fig. 2). The reactive iron pool, expressed in fluorescence units, was 562 ± 47; 675 ± 46; 893 ± 83; and 1180 ± 115 for cells cultured in 5 μM, 20 μM, 50 μM and 100 μM, respectively. Thus, increasing the concentration of iron in the extracellular medium resulted in increased levels of total intracellular iron and increased levels of the reactive iron pool. These findings are in agreement with previous observations in Caco-2 cells, where increasing concentrations of iron in the culture media elicited increases in the size of the labile iron pool (Núñez *et al*. 2001).

Iron-mediated activation of IRP1 activity

Increasing levels of intracellular iron should inhibit IRP activity, shutting-off the iron uptake process. As iron uptake did not shut-off after 4 days in culture (Fig. 1), we investigated the activity of the IRP proteins, which should determine the cellular levels of TfR, the major protein involved in iron uptake. Neuroblastoma cells and hippocampus neurons had an active IRE/IRP system, as determined by a band-shift assay (Fig. 3). IRP1 and IRP2 activity was differentiated by β-mercaptoethanol activation (IRP1) and by the super-shift induced by an IRP2-specific antibody (Fig. 3a). Increasing the iron concentration in the culture media from 1.5 to 20 μM resulted in the decrease of both IRP1 and IRP2 activity (Fig. 3a). A further increase of extracellular iron concentration to 40 μM abolished IRP2 activity. Paradoxically, IRP1 activity increased when cells

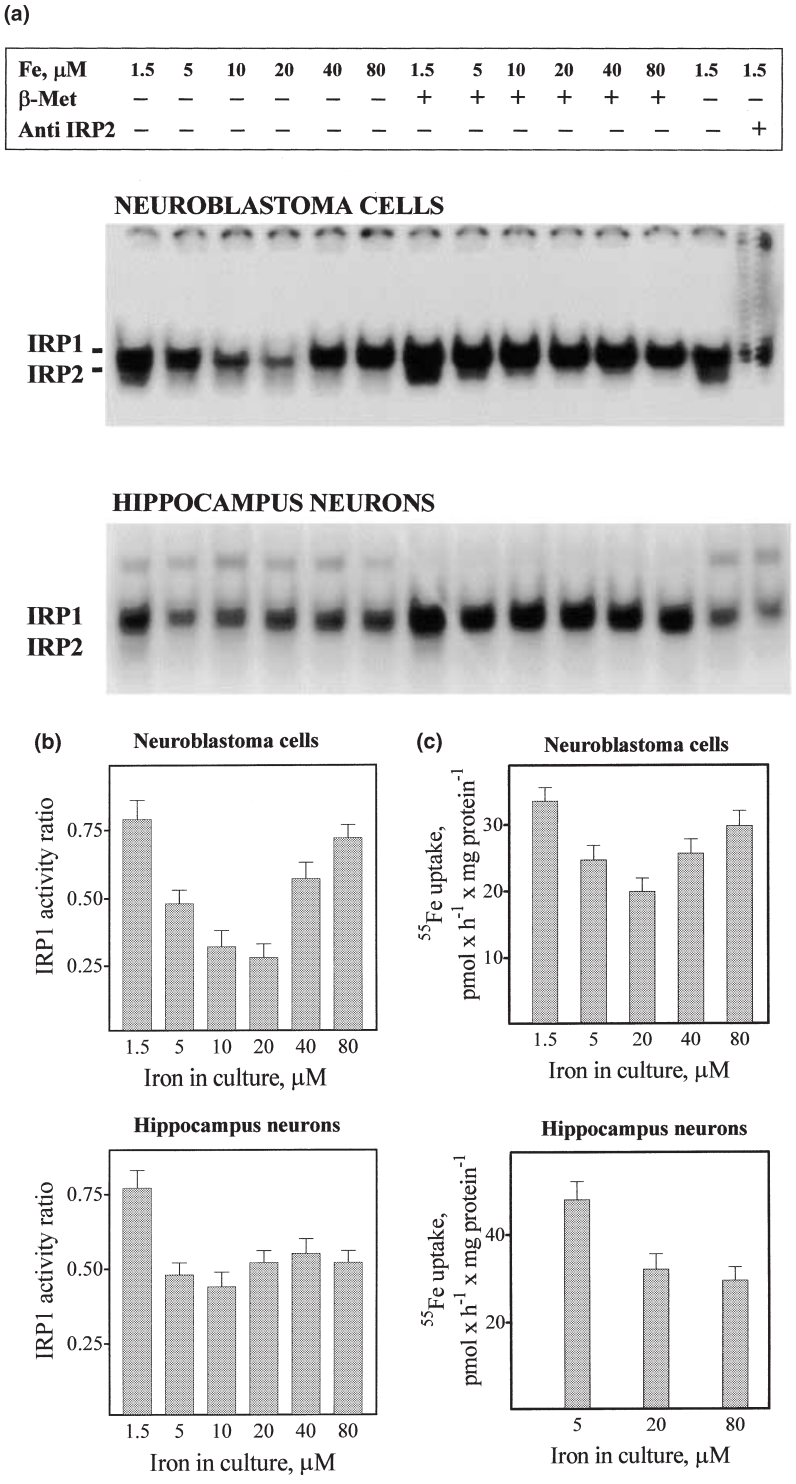

Fig. 3 IRP activity in cells cultured in increasing concentrations of iron. (a) Neuroblastoma cells or hippocampal neurons were incubated for 2 days in a media containing 1.5, 5, 10, 20, 40, or 80 μM Fe. IRE-binding activity was the determined in cell extracts using a Phosphoimager device, as described in Methods. The positions of IRP1 and IRP2 are indicated on the right. IRP1 was identified by its activation with β-mercaptoethanol, while IRP2 was identified by its shift with anti-IRP2 antibody. (b) IRP1 activities shown in (a) were further quantified by dividing IRP1 activity in the absence of β-mercaptoethanol by the activity in the presence of β-mercaptoethanol. The activity of the individual bands was determined by density analysis using Quantity One software (Bio-Rad). (c) Neuroblastoma cells or hippocampal neurons, previously equilibrated for 2 days with the iron concentrations shown in the abscissa, were incubated in quadruplicates for 60 min in MOPS–saline pH 7.0 supplemented with 10 μM $^{55}Fe^{3+}$ as the complex $^{55}FeCl_3$–NTA. Cells-associated ^{55}Fe was then determined in cell extracts. ^{55}Fe uptake was expressed as pmol of ^{55}Fe/h/mg of protein.

were incubated in 40 and 80 μM iron (Fig. 3b). This behavior of IRP1 was reproducibly seen in three independent assays. IRP2 activity of hippocampal neurons was abolished at iron concentrations of 20–40 μM (Fig. 3a), while IRP1 activity diminished with iron concentrations up to 10 μM and then remained constant, at about 50% the activity elicited by β-mercaptoethanol (Fig. 3b).

^{55}Fe uptake by neuroblastoma cells and hippocampal neurons closely followed IRP1 activity. In N2A cells, iron uptake decreased when iron increased from 1.5 μM to 20 μM

in the culture media, and then increased when iron in the culture raised to 40 μM and 80 μM (Fig. 3c). In hippocampal neurons, iron uptake decreased up to 20 μM iron and then remained constant up to 80 μM iron in the culture media (Fig. 3c). Therefore, induction of IRP1 activity was accompanied by the induction of the iron uptake process.

As cellular iron can give rise to reactive oxygen species, we tested the hypothesis that the induction of IRP1 activity was mediated by oxidative stress. When N2A cells were cultured with 40 μM Fe and with varied concentrations of the antioxidant *N*-acetyl-L-cysteine (NAC), a dose-dependent decrease in IRP1 activity was observed (Fig. 4a). Quantification of the change of IRP1 activity by density analysis, indicated a threefold decrease when the cells were incubated with 5–10 mM NAC (Fig. 4b). Therefore, the effect of iron on IRP1 activity was most probably mediated by oxidative stress. An equivalent arrest of iron-mediated IRP1 activation was obtained with the flavonoid quercetin in the 5–150 μM range (data not shown). NAC also decreased the stimulation of IRP1 activity by extracellular H_2O_2 (Pantopoulos *et al.* 1997), hence, it is possible that iron and H_2O_2 follow similar oxidative stress-mediated intracellular signaling pathways to activate IRP1.

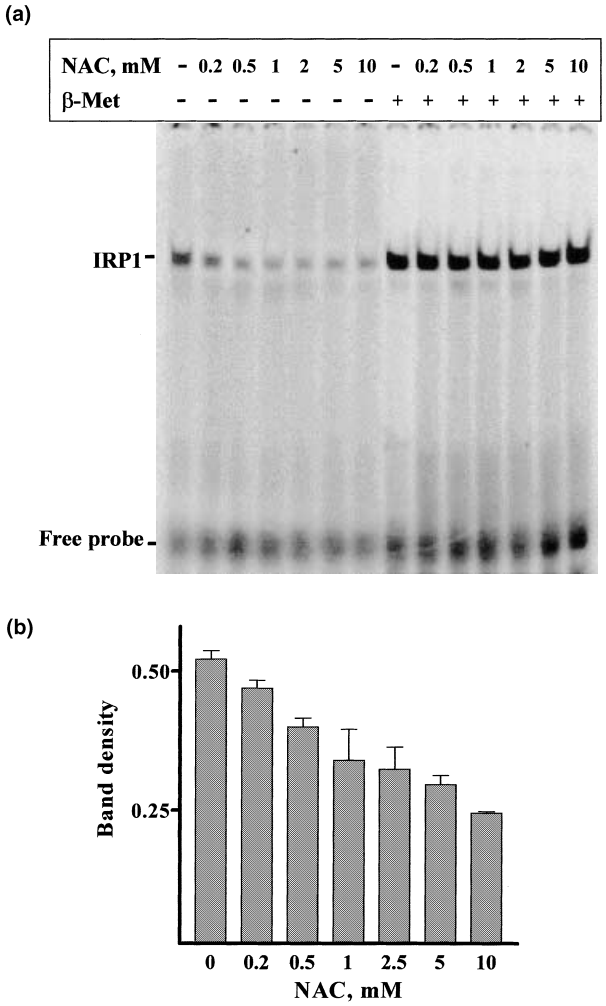

Fig. 4 *N*-acetyl-L-cysteine arrests iron-induced IRP1 activity. (a) A representative band-shift assay of IRP activity of N2A cells stimulated for 2 days with 40 μM Fe in the presence of the stated concentrations of NAC. An upper-limit concentration of 10 mM NAC was selected because incubation with 20 and 30 mM NAC (Pantopoulos *et al.* 1997) produced cell toxicity under the assay conditions used here. (b) IRP1 activity shown in (a) was further quantified by dividing IRP1 activity in the absence of β-mercaptoethanol by the activity in the presence of β-mercaptoethanol. The activity of the individual bands was determined by density analysis using Quantity One software (Bio-Rad). Shown are means ± SD from two independent experiments.

We have observed that physiological concentrations iron induce oxidative damage in intestinal epithelial cells (Núñez *et al*. 2001). Accordingly, we examined the ability of physiological concentrations of iron to induce oxidative damage to neuroblastoma and hippocampal cells. To test for iron-induced damage to proteins in neuroblastoma cells, we determined the formation of HNE adducts as a function of iron in the culture medium. Immunoreactivity to monoclonal antibody HNEJ-2 revealed that increasing amounts of HNE adducts was generated when iron in the culture media increased from 1 to 5 μM (Fig. 5). However, little change in the pattern of HNE-containing proteins was observed in the range 5–80 μM Fe in the culture media. Moreover, no loss of cell viability was observed under these conditions, as assessed by the DCF fluorescence and the MTT assays (not shown).

Fe-mediated oxidative damage to DNA was assessed with monoclonal antibody N45.1, which recognizes 8-OHdG, a highly specific product of DNA damage. Immunostaining

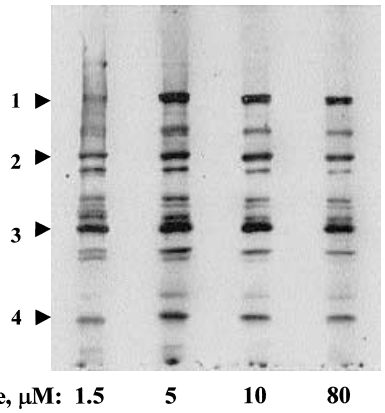

Fig. 5 Oxidative damage to proteins determined by HNE-immunoreactivity. Extracts from neuroblastoma cells cultured for 2 days in 1.5, 5, 10 or 80 μM Fe were separated by sodium dodecyl sulphate gel electrophoresis. The proteins were blotted into nitrocellulose and the HNE–protein adducts were detected with monoclonal antibody HNEJ-2. Arrowheads denote proteins that were most modified by iron increases. Shown is one of three similar results.

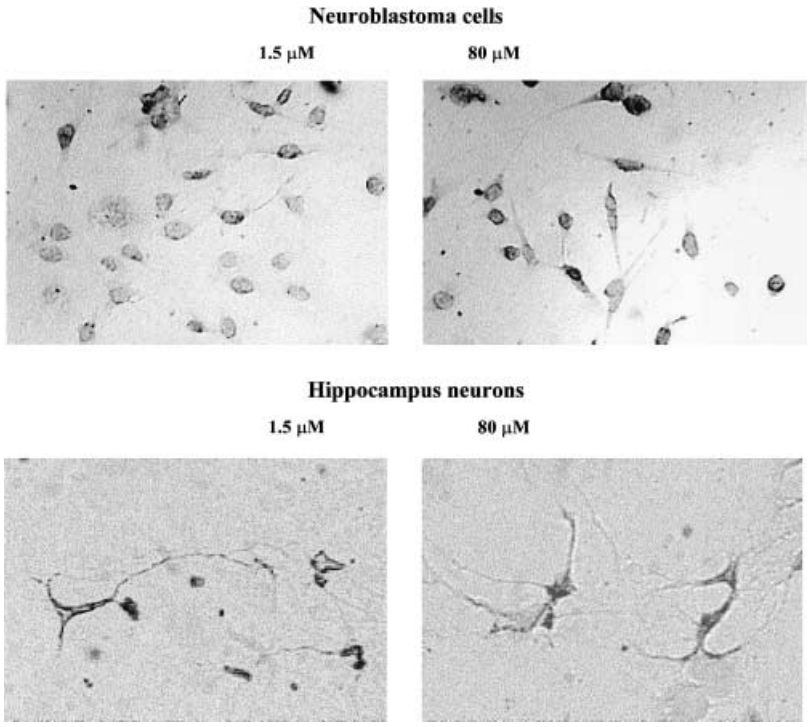

Fig. 6 Iron-induced DNA damage. Neuroblastoma cells and hippocampal neurons were cultured for 2 days either in 1.5 or 80 μM Fe media, and then were immunostained for 8-OHdG to detect DNA damage. A darker staining indicates increased 8-OHdG immunoreactivity. In no case was loss of cell viability observed. Shown are representative pictures of N2A and hippocampal cells showing a mild increase in oxidative damage at high iron concentrations.

neuroblastoma cells or hippocampus neurons cultured for 2 days with high (80 μM) iron revealed a mild increase in 8-OH–DNA immunoreactivity when compared with cells cultured in 1.5 μM Fe (Fig. 6). Therefore, although no loss of cell viability was observed with up to 80 μM iron in the culture media, iron generated oxidative damage to DNA.

Discussion

Using neuroblastoma cells and hippocampal neurons in culture, we found that intracellular iron accumulation did not prevent further iron uptake by these cells. Moreover, not all the cellular iron was safely stored in ferritin, as the reactive iron pool increased as the iron in the culture media increased. We have found a similar pattern, i.e. lack of a total shut-off of Fe uptake at high iron concentrations, in Caco-2 intestinal cells but, contrary to this study, Caco-2 cells suffered considerable oxidative damage at 40–80 μM iron (Núñez *et al*. 2001). Similarly, we have observed continuous iron uptake by HepG2 cells, despite a large iron load (M. T. Núñez, unpublished results). These findings underscore the need to understand the functioning of the specific components of the iron homeostasis system based on cell-type.

Iron regulatory proteins are the central components of the mechanism by which mammal cell maintain cellular iron homeostasis. We found that N2A cells had both IRP1 and IRP2 activities, but interesting differences were noted in their responses to iron. IRP2 activity, which was considerably less active than IRP1, was effectively abolished when the extracellular concentration of iron was raised from 1.5 to 80 μM Fe. On the contrary, although IRP1 activity decreased when the extracellular concentration of iron was raised from 1 to 20 μM, further increases in the extracellular iron concentration to 40 and 80 μM resulted in a paradoxical increase (N2A) or maintenance (hippocampal neurons) of IRP1 activity. These data can be interpreted in terms that at high iron concentrations IRP1 becomes resistant to iron-mediated inactivation. Because the activation of IRP1 by oxidants like H_2O_2 has been documented (Martins *et al*. 1995; Pantopoulos *et al*. 1997), this increase could be mediated by iron-induced oxidative stress. In agreement with this reasoning, we found that the activity of IRP1 in cells cultured in 40 μM iron decreased considerably when the antioxidant NAC was also present in the culture medium. Activation of IRP1 by H_2O_2 requires a membrane-associated cell component as H_2O_2 does not activate IRP1 in cytosolic extracts (Martins *et al*. 1995; Pantopoulos and Hentze 1998). These observations have led to the proposal that a signaling system mediates the extracellular action of H_2O_2 (Pantopoulos and Hentze 1998). Both H_2O_2 (Pantopoulos *et al*. 1997) and Fe-mediated activation of IRP1 (this paper) were blocked by NAC. Thus, part of the putative oxidative stress-mediated signal transduction route that leads to the activation of IRP1 could be common both for H_2O_2 and for iron.

Increases in IRP1 activity were accompanied by increases in cell iron uptake, an indication that IRP1 was functional in

the activation of the iron uptake system. Intriguingly, this iron-induced iron uptake feedback loop results in more intracellular iron, which should result in increased Fenton-mediated oxidative stress. Increased oxidative stress could, in turn, result in apoptotic cell death (Kawabata *et al*. 1997; Castagne *et al*. 1999; Ray *et al*. 2000). The question arises as to why such a mechanism should exist. Active cellular suicide by apoptosis plays a significant role in animal development, tissue homeostasis and a wide variety of diseases, including cancer, AIDS, stroke and many neurodegenerative disorders (Song and Steller 1999). It is possible that such a mechanism could be used in early organogenesis, during which massive apoptosis ensue (Raff 1998; Hilger-Eversheim *et al*. 2000). It could also be a mechanism of cell suicide, by which cells that can no longer maintain an adequate redox state enter a pro-oxidant loop that finally ends in apoptosis or necrosis.

Corresponding with undeterred iron uptake, cells grown in a medium with a high but physiological content of iron presented oxidative damage to proteins and DNA, an indication that cell iron accumulation, with the accompanying increase in the labile iron pool, was the direct cause of the oxidative damage. A net increase in HNE–protein adducts was observed when the extracellular iron concentration was raised from 1 to 5 μM. Surprisingly, further increases in iron concentrations did not promote further damage to proteins. These findings contrast with the considerable Fe-mediated damage found in Caco-2 cells, where evident signs of cell disintegration were found in cells cultured in 50 μM Fe (Núñez *et al*. 2001). It is apparent from the above data that neuroblastoma cells and hippocampal neurons respond to iron-induced oxidative stress generating effective protection mechanisms that avoid gross oxidative cell damage. Nevertheless, with time, the increased oxidative load generated by iron accumulation could become unmanageable and result in cell death.

In summary, iron uptake activity by neuroblastoma cells and hippocampal neurons decreased when iron in the culture media increased from 1.5 to 20 μM. At iron concentrations of 40 μM and higher, iron uptake actually increased. This was most probably due to iron-induced oxidative activation of IRP1. Iron accumulation by the cells correlated to oxidative damage to proteins and DNA. The process described here of iron-induced, oxidative stress-mediated increase of iron uptake may underlie some processes associated with neuronal degeneration and death.

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