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Iron-activated iron uptake: A positive feedback loop mediated by iron regulatory protein 1

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Abstract

The love-hate relationship between iron and living matter has generated mechanisms to maintain iron concentration in a narrow range, above and below which deleterious effects occur. At the cellular level, iron homeostasis is accomplished by the activity of the IRP proteins, which, under conditions of iron depletion, up-regulate the expression of the iron acquisition proteins TfR and DMT1. It has been shown that hydrogen peroxide activates IRP1 and that this activation mediates a potentially harmful increase in cell iron uptake. Here we show that IRP1 activity is also induced by iron-mediated oxidative stress. When cells were incubated with up to 20 μM of iron, a typical decrease in IRP1 and IRP2 activity was observed. Interestingly, when iron was further increased to 40 or 80 μM, IRP1 was reactivated in three of the four different cell lines tested, i.e., Caco-2 cells, N2A cells and HepG2 cells. In the fourth cell line (K562) IRP1 activity did not increase, but neither did it decrease. This response to iron was largely abrogated when the antioxidant N-acetyl cysteine was added along with iron to the culture medium. Thus, the effect of iron was mediated by oxidative stress. Increases in IRP1 activity were accompanied by increases in cell iron uptake, an indication that the activated IRP1 was functional in the activation of iron uptake. Hence, this iron-induced iron uptake feedback loop results in the increase of intracellular iron and increased oxidative stress.

Introduction

The pathophysiology of transition metals is receiving increasing attention due to their ability to generate reactive oxygen species and oxidative damage under physiological conditions. In particular, iron and copper have been involved in the generation of oxidative damage in several neurodegenerative diseases such as Parkinson, Alzheimer and amyotropic lateral sclerosis (reviewed by Sayre *et al.* 2000). Through the Fenton reaction, Fe^{2+} or Cu^{1+} generate the highly reactive hydroxyl radical, which quickly abstracts H-atoms from most organic molecules (Symons & Gutteridge 1998). Because of its non-enzymatic nature, the production of the hydroxyl radical through the Fenton reaction is directly proportional to the cellular concentration of reactive iron or copper (Okada 1996).

Iron was first implicated in carcinogenesis in autopsy studies of individuals with hemochromatosis, an iron metabolism disorder characterized by increased body iron absorption (Warren & Drake 1951; Ammann *et al.* 1980; Bradbear *et al.* 1985). With time, evidence accumulated establishing that iron and copper are both toxic and carcinogenic (reviewed in Okada, 1996, 1998). Recent trials using iron chelators against a number of aggressive cancers have reported some arrest in tumor growth (Richardson 1997), an indication that iron can have a neoplastic action, although they could merely be sequestering iron from ribonucleotide reductase, an enzyme essential for cell proliferation. The intimate mechanisms of iron- and copper-induced carcinogenesis are still not understood, but mounting evidence indicates that iron toxicity is mediated by its

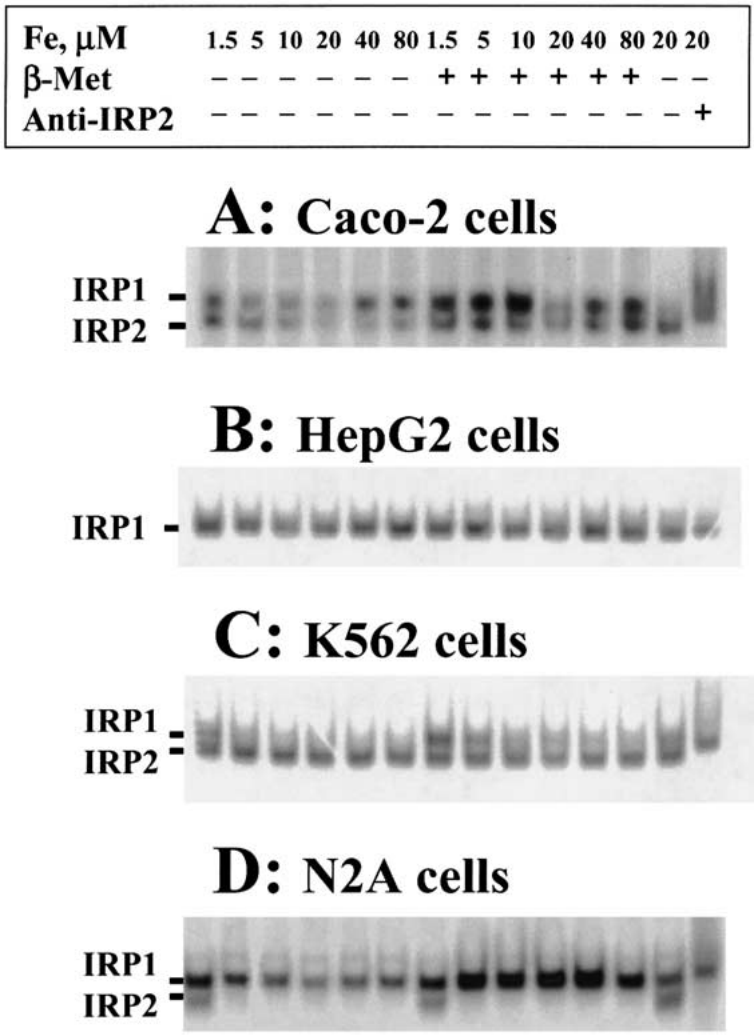

Fig. 1. Band-shift assay of cells cultured with varied concentrations of iron. Caco-2, HepG2, K562 and N2A cells were incubated for 2 days in a media containing 1,5, 5, 10, 20, 40, or 80 μM Fe. IRE binding activity was the determined in cell extracts using a Phosphoimager device, as described in the Methods section. 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.

ability to produce free radicals (reviewed in Okada 1998). Nevertheless, substantial opinions have been advanced stating that iron plays no major role in tissue damage or in carcinogenesis (Weinbren *et al.* 1978; Walker & Segal 1999; MacDonald *et al.* 1999).

Iron is an essential element for life. Therefore, despite its potential toxicity, mechanisms have 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) (Kuhn & 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 the TfR, as it has an IRE motif functional in IRP binding (Wardrop & 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 condi-

tion, IRP1 has a 4S-4Fe cubane that renders the protein aconitase-active but inactive for IRE-binding. Low levels of intracellular Fe induce a disassembling of the 4S-4Fe cluster, which causes IRP1 to bind to and stabilize TfR mRNA and to bind to ferritin mRNA, diminishing its translation (Kuhn & Hentze 1992; Kim *et al.* 1995; reviewed in Eisenstein 2000; Thiel & 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, hydrogen peroxide, hypoxia and phosphorylation (Hanson *et al.* 1999) also regulate IRPs. IRP1 is activated by extracellular 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 increased expression of the transferrin receptor (TfR) and increased cell iron uptake (Caltagirone *et al.* 2001), a response that could deregulate cell iron homeostasis.

Based on iron's property of inducing oxidative stress, in our present work we analyzed whether iron could induce the activity of IRP1, a paradoxical response that could induce sustained iron uptake and iron-mediated oxidative damage. We found that high intracellular levels of iron indeed activated IRP1, accompanied by sustained iron uptake and oxidative damage. We hypothesize that iron activation of IRP1 could be part of a suicidal mechanism in cells whose iron-homeostasis capacity has been surpassed.

Materials and methods

Materials

Fetal bovine serum, transferrin (Tf), culture medium, protease inhibitors, culture media, buffers and salts were purchased from Sigma Chem. Co. (St. Louis, MO). The membrane-permeant iron chelator 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. 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. ^{55}Fe in the ferric chloride form was from New England Nuclear (Boston, MA). Culture plasticware and Transwell bicameral inserts were from Corning Costar (Cambridge, MA).

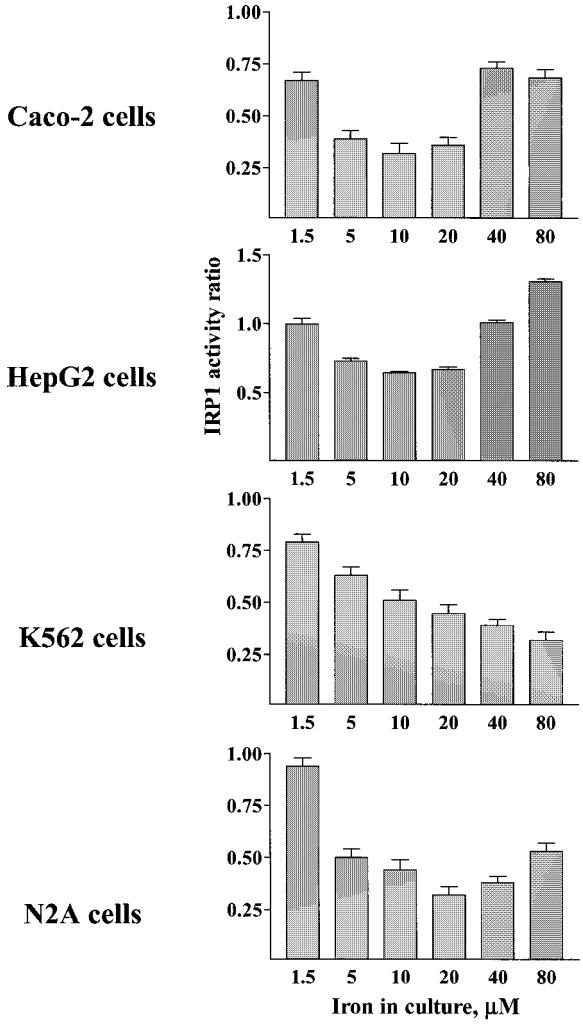

Fig. 2. Quantification of IRP1 activity in cells cultured with varied concentrations of iron. IRP1 activity shown in Figure 1 was further quantified by dividing IRP1 activity in the absence of β-mercaptoethanol by the activity in the presence of β-mercaptoethanol. The activity of the individual bands was determined by density analysis using the Quantity One software (Bio-Rad).

Cell culture

Mouse neuro-2a (N2A) cells, human hepatoma HepG2 cells, human intestinal epithelia Caco-2 cells and human granulocytic K562 cells were from the American Type Culture Collection (CCL-131, Rockville, MD). The cells were cultured in DMEM supplemented with 5% fetal bovine serum (N2A cells) or 10% fetal bovine serum (Caco-2 cells, HepG2 cells and K562 cells). To induce neuronal differentiation, N2A cells were cultured with 0.2 mM dibutyryl cAMP (Chen *et al.* 1983). The culture medium was changed

every 2 days. Cells were trypsinized and replated every 6–7 days. After 6 days in culture, N2A cells started to detach from the dish, so time-based experiments were limited to a maximum of 6 days.

When challenged with varied concentrations of iron, cells were trypsinized and grown for 2 days (N2A cells) or 4 days (Caco-2 cells, HepG2 cells and K562 cells) in DMEM, 10% 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 1.5, 10, 20, 40 or 80 μM Fe^{3+} as the complex FeCl3-sodium nitrilotriacetate (NTA, 1:2, mol:mol). The 80 μM iron concentration corresponds to a condition of 100% plasma Tf saturation (40 μM diferric Tf), while the 40 μM iron condition corresponds to a 50% plasma Tf saturation. In contrast, Tf saturation in normal plasma is approximately 30%. Although the 40 μM and the 80 μM iron conditions were not in the physiological range, this *in vitro* condition allowed for an accelerated modification of cell iron status in this study.

Cell extracts

Cell extracts were prepared by treating cells with lysis buffer (50vμl per 1×10^6 cells of 10 mM HEPES, pH 7.5, 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 and sedimented for 10 min at $10,000 \times g$. Protein in the supernatant was determined by the Lowry method. The supernatant was stored at -70 °C.

Band-shift assay

IRP activity was determined by a RNA band-shift assay performed as described earlier (Leibold & Munro 1988; Arredondo *et al.* 1997). β-mercaptoethanol was used at 2% (vol./vol.) 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). 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 the Quantity One software (Bio-Rad).

Western immunodetection

A polyclonal antibody against DMT1 was raised in rabbits against the peptide SKGLLTEEATRGYVK, corresponding to the carboxyl-terminal segment of DMT1 (Arredondo *et al.* 2001). DMT1, from 20 μg of cell extract protein, was detected by Western blot analysis, using a chemoluminiscence kit (SuperSignal, Pierce Chem. Co.). Primary and secondary antibodies were used at 1:1,000- and 1:20,000-fold dilutions, respectively.

^{55}Fe uptake

Cells that were previously cultured for 2 days with the iron concentrations shown in the abscissa were incubated in triplicates for 60 min in MOPS saline (20 mM MOPS-OH, 150 mM NaCl, 1.8 mM $CaCl_2$, 5 mM glucose, pH 7.0) supplemented with 10 μM $^{55}Fe^{3+}$ as the complex $^{55}FeCl_3$-NTA. Cell-associated ^{55}Fe was then determined in cell extracts. Cell uptake was expressed as pmol of ^{55}Fe/h/mg of protein.

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

Reasoning that iron could induce oxidative stress and that oxidative stress could induce IRP activity, we first studied the response of the IRE/IRP system to increasing concentrations of iron in the culture media (Figure 1). IRP1 and IRP2 were differentiated by their activation by β-mercaptoethanol (IRP1) and the super-shift induced by an IRP2-specific antibody. Increasing the iron concentration in the culture media from 1.5 to 20 μM resulted in an apparent decrease of IRP1 activity in Caco-2 (Figure 1A), HepG2 (Figure 1B), K562 (Figure 1C), and N2A cells (Figure 1D). Paradoxically, a further increase of extra-cellular iron concentration to 40 and 80 μM induced an increase of IRP1 activity in Caco-2, N2A and HepG2 cells.

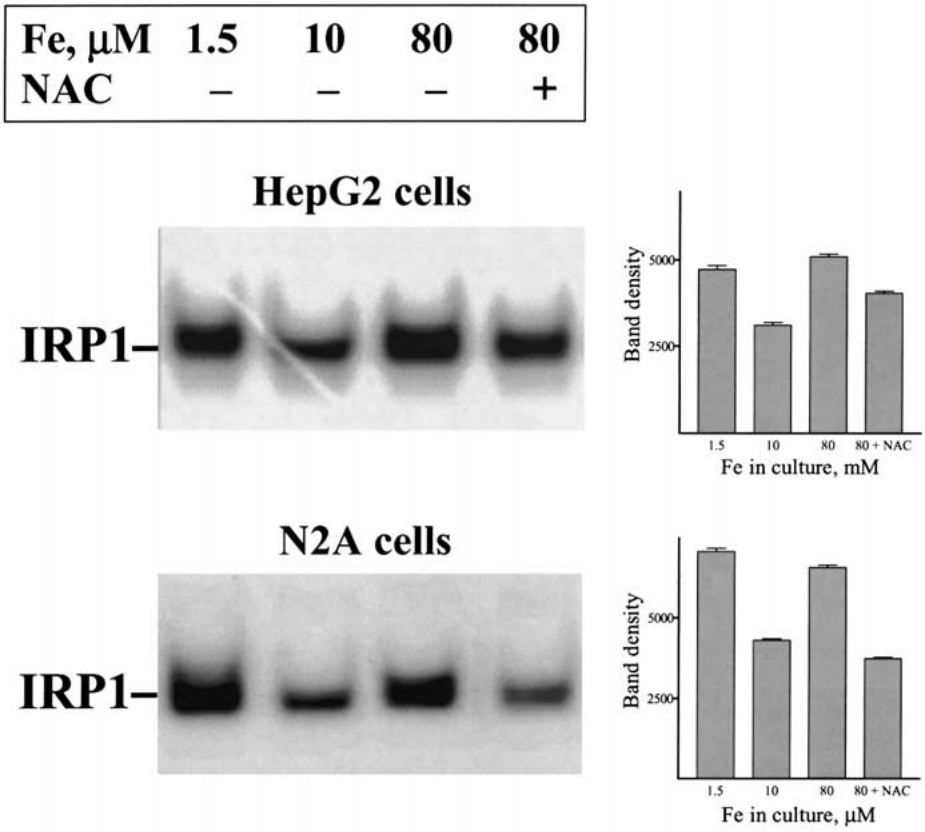

Fig. 3. The anti-oxidant NAC blocks Fe-induced activation of IRP1. HepG2 or N2A cells were incubated for 2 days with 1,5, 10 or 80 μM Fe, or with 80 μM Fe in the presence of 10 mM NAC. IRE binding activity was then determined in cell extracts as described in Materials and methods. Shown on the left is the quantification of IRP1 activity by density analysis. Ten mM NAC reduced 80 μM iron IRP1 activation by 20 and 42%, in HepG2 and N2A cells, respectively.

This behavior of IRP1 was reproducibly observed in three independent assays. The effect was cell type-dependent since we did not observe IRP1 activation in K562 cells, although IRP1 activity remained at a discrete level even at 80 μM iron. Figure 2 shows a density band quantification of the results in Figure 1, normalized by IRP1 activity in the presence of β-mercaptoethanol as a load control. Increasing iron from 20 to 80 μM, resulted in an IRP1 activation of 1.95, 1.75, and 1.41-fold, for, Caco-2, HepG2, and N2A cells, respectively.

We previously showed that iron induces oxidative stress in Caco-2 cells (Núñez *et al.* 2001). This observation, plus the reported activation of IRP1 by hydrogen peroxide (Martins *et al.* 1995; Pantopoulos *et al.* 1997), prompted us to test the role of iron-induced oxidative stress on IRP1 activation. To do that, we determined the response of N2A and HepG2 cells to iron when co-incubated in the presence of N-acetyl cysteine, a free radical scavenger and a glutathione precursor (Figure 3). The cells were incubated for 48 h with varied concentrations of iron in the culture media. NAC (10 mM) was also present at the concentrations of iron that induced IRP1 (40 and 80 μM Fe). As observed earlier, IRP1 activity diminished when iron increased from 1.5 μM to 20 μM and then increased when iron in the culture rose to 40 μM and 80 μM. The presence of NAC under the last two conditions considerably reduced the iron-mediated activation of IRP1 in HepG2 cells (Figure 3). Similar results were obtained with Caco-2 cells and N2A cells (data not shown). NAC also stabilized IRP2 activity (Figure 3), probably by inhibiting the iron-mediated proteasome degradation of IRP2 (Iwai *et al.* 1998).

Having established that iron in the 40–80 μM range induced IRP1 activity, we then examined cellular iron uptake, reasoning that the observed activation of IRP1 should result in the expression of proteins in-

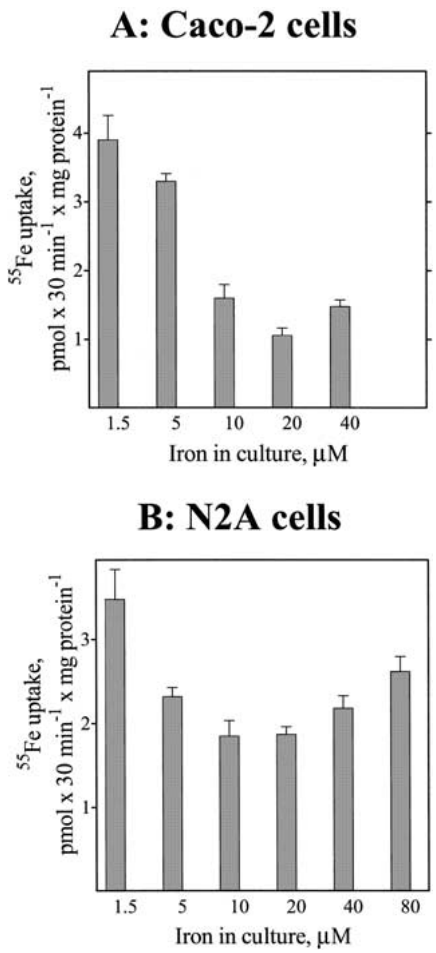

Fig. 4. ^{55}Fe uptake by cells cultured in varied iron concentrations. N2A or Caco-2 cells, previously equilibrated for 2 days with the iron concentrations shown in the abscissa, were incubated in quadruplicates for 60 min in MOPS saline (20 mM MOPS-OH, 150 mM NaCl, 1.8 mM $CaCl_2$, 5 mM glucose, pH 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.

volved in iron uptake. To that end, N2A and Caco-2 cells were equilibrated for 2 days with varied concentrations of iron in the culture media and then tested for ^{55}Fe uptake. Both N2A and Caco-2 cells uptake closely followed IRP1 activity, i.e., ^{55}Fe uptake decreased when iron increased from 1.5 μM to 10–20 μM in the culture media, and then increased when iron in the culture rose to 40 μM and 80 μM Figure 4). Thus, iron not only induced IRP1 activity, but it also induced the iron uptake machinery.

Discussion

The existence of two stable forms of iron, iron(III) and iron(II), allows both for one-electron transfer reactions vital to life, and for the generation of free radical species. In particular, in the presence of peroxides, Fe(II) promotes the formation of the hydroxyl free radical, one of the most reactive chemical species with the ability to attack lipids, proteins and DNA (Symons & Gutteridge 1998). The reactivity of the hydroxyl radical leads to cell death, cellular necrosis, apoptosis, mutation and malignant transformation (Okada 1998). This love-hate relationship between iron and living matter has generated mechanisms to maintain iron concentration in a narrow range, above and below which deleterious effects occur. At the cellular level, iron homeostasis is accomplished by the activity of the IRP proteins, which, under conditions of iron depletion, up-regulate the synthesis of the iron acquisition proteins TfR and DMT1.

It was shown previously that hydrogen peroxide activates IRP1 (Martins *et al.* 1995; Pantopoulos *et al.* 1997), and that this activation mediates a potentially harmful increase in cell iron uptake (Caltagirone *et al.* 2001). Therefore, oxidative stress activates IRP1 by unknown mechanisms. Here we show what could be a related process, i.e., the iron-induced activation of IRP1. This mechanism is most probably based on the activation of IRP1 by iron-induced oxidative stress. When cells were incubated with up to 10–20 μM iron, a typical decrease in IRP1 and IRP2 activity was noted. Interestingly, when iron was further increased to 40 or 80 μM, IRP1 was reactivated in three of the four cell lines tested, i.e., Caco-2 cells, N2A cells and the HepG2 cell. In a fourth cell line IRP1 activity did not increase, but likewise did not decrease any further either. This response to iron was largely abrogated when the antioxidant N-acetyl cysteine was also added to the culture medium. Thus, the effect of iron was mediated by oxidative stress.

Increases in IRP1 activity were accompanied by increases in cell iron uptake, an indication that the activated IRP1 was functional in the activation of the iron uptake system. Hence, this iron-induced iron uptake feedback loop results in more intracellular iron and increased oxidative stress. Increased oxidative stress could in turn result in apoptotic cells death (Castagne *et al.* 1999; Kawabata *et al.* 1997; Ray *et al.* 2000). The question arises as to why such a mechanism should exist. Active cellular suicide by apoptosis plays important roles in animal development, tissue

homeostasis and a wide variety of diseases, including cancer, AIDS, stroke and many neurodegenerative disorders (Song & Steller 1999). It is possible that such a mechanism could be used in early organogenesis, during which massive apoptosis occurs (Hilger-Eversheim *et al.* 2000; Raff 1998). It could also be a mechanism of cell suicide, by which cells that can no longer maintain an adequate redox state enter a positive pro-oxidant loop that finally ends in apoptosis or necrosis. The two processes are not opposites and could be used in states of life as different as early development and cell death.

Acknowledgements

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