FEBS Letters 382 (1996) 304–308 FEBS 16804

Dynamics of the cytosolic chelatable iron pool of K562 cells

William Breuer*, Silvina Epsztejn, Z. Ioav Cabantchik

Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, 91904, Israel

Received 13 February 1996

Abstract The labile iron pool of cells (LIP) constitutes the primary source of metabolic and catalytically reactive iron in the cytosol. We studied LIP homeostasis in K562 cells using the fluorescent metal-sensitive probe calcein. Following brief exposure to iron(II) salts or to oxidative or reductive stress, LIP rose by up to 120% relative to the normal level of 350 nM. However, the rate of recovery to normal LIP level differed markedly with each treatment (respective $t_{1/2}$s of 27, 65–88 and ≤17 min). We show that the capacity of K562 cells to adjust LIP levels is highly dependent on the origin of the LIP increase and on the pre-existing cellular iron status.

Key words: Iron; Oxidative stress; Fluorescence; Calcein; Chelators

1. Introduction

Current models of iron acquisition, sequestration and storage by mammalian cells invoke regulated adjustments in the levels of membrane Tf receptors and cytosolic ferritin. The cell iron form which apparently reflects the iron status of cells has been associated with a weakly bound, low-molecular-weight fraction [1–3] which is also the presumed target of iron chelators [4–8]. In the prevailing models, the chelatable or labile iron pool (LIP) is assumed to be sensed by a cytosolic iron-responsive protein (IRP) which coordinately represses ferritin mRNA translation and increases transferrin (Tf) receptor mRNA stability [11]. However, the implied (inverse) relationship between LIP and IRP activity [12] remains to be experimentally established as does its involvement in short-term adjustments of LIP. These issues are of physiological relevance, since it is generally assumed that LIP must be under strict homeostatic control not only for long-term metabolic purposes, but for minimizing the potentially damaging effects of LIP [9]. This assumption is based primarily on the correlation found between cellular resistance to oxidative stress and depletion of cell iron pools induced by iron chelators [4,5,9,13]. In this work we provide experimental evidence for the existence of rapidly acting homeostatic mechanisms for maintaining LIP at stable levels. Changes in the level of the cytosolic iron pool were followed continuously by a non-invasive method which we developed for in situ monitoring of LIP using the fluorescent metal-sensitive probe calcein, CA [3,14].

*Corresponding author. Fax: (972) (2) 6586974.
E-mail: IOAV@VMS.HUJI.AC.IL

Abbreviations: CA, calcein; CA-AM, calcein acetoxymethyl ester; LIP, intracellular labile iron pool; Tf, transferrin; SIH, salicylaldehyde isonicotinoyl hydrazone; FAS, ferrous ammonium sulfate; FCS, fetal calf serum; ME, β-mercaptoethanol; TBHP, *tert*-butylhydroperoxide.

2. Materials and methods

2.1. Materials

CA and its acetoxymethyl ester (CA-AM) were obtained from Molecular Probes (Eugene, OR). The divalent metal ionophore A23187, ferrous ammonium sulfate (FAS), ferric ammonium citrate (FAC) were from Sigma Chem. Co. (St. Louis, MO). All other materials were of highest available grade. The iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) (a generous gift from Dr. P. Ponka, Lady Davis Institute for Medical Research, Montreal, Canada) was prepared as 50 mM stock solution in dimethyl sulfoxide. Chromatographically pure Tf was from Kama-Da Industries (Kibbutz Kama, Israel).

2.2. Cell treatments and loading with calcein

Human erythroleukemia K562 cells were propagated in α-MEM medium containing 7% fetal calf serum supplemented with L-glutamine and antibiotics. Manipulations of the cellular iron status were achieved by culturing the cells for 18 h in α-MEM medium containing 1 mg/ml bovine serum albumin with added 50 μg/ml human Tf (iron-replete) or 100 μM FAC (iron-overloaded) or no addition (iron-starved). A modification of the previously published [3] method of CA loading and LIP determination was used. Cells were loaded at a density of 1×10^7 cells/ml with 0.25 μM CA-AM for 5 min at 37°C in HEPES-buffered, bicarbonate-free α-MEM medium containing 1 mg/ml BSA, 20 mM HEPES, pH 7.3 (α-MEM-HEP-BSA), washed of excess CA-AM, resuspended in α-MEM-HEP-BSA medium to 1×10^7 cells/ml and maintained at room temperature until used. Just prior to measurements, 1 ml of the CA loaded cell suspension was centrifuged in a microcentrifuge and the cells were resuspended in 2 ml of pre-warmed 150 mM NaCl, 20 mM HEPES, pH 7.3 (HBS buffer). The cell suspension was transferred to a stirred, thermostatted (37°C) cuvette and CA fluorescence was measured (excitation 488 nm, emission 517 nm, slits 10 nm, Perkin-Elmer LS-5B fluorometer equipped with a temperature-controlled cuvette holder and magnetic stirrer). A fluorescence quenching, anti-CA antibody (10 μl/cuvette) was added in all experiments to eliminate extracellular fluorescence, which amounted to approx. 5% of the total. The concentration of free CA accumulated inside cells under the above loading conditions was 9 μM, determined as in [3].

2.3. Estimation of cellular chelatable iron content with calcein and a membrane permeant iron chelator

A baseline fluorescent signal was obtained for a suspension of CA-loaded cells and the signal was adjusted to 70 (in a scale of 0–100). The increase in the fluorescence produced by the addition of the highly permeable iron chelator, SIH, 100 μM, was recorded. Since CA is insensitive to Ca^{2+} and Mg^{2+} up to 1 mM [14], and the intracellular concentration of CA-quenching metals other than iron (Cu^{2+}, Co^{2+} and Ni^{2+}) is very low, we conclude that the signal represents CA-bound iron [3,14].

Calibrations to determine the relationship between changes in fluorescence and intracellular iron concentration were carried out by adding 10 μM ionophore A23187 (this produced a <5% change in the signal), followed by 0.5 μM iron(II) added as FAS, and determining the corresponding change in the fluorescence. This calibration relies on the assumption that in the presence of A23187 iron(II) is fully equilibrated across the cell membrane so the CA-detectable concentration of intracellular iron is 0.5 μM.

2.4. Cell viability

CA has also been used extensively as a vital dye [32]. Since CA is retained only by viable cells, and a fluorescence-quenching, anti-CA antibody was present in the medium during all determinations (in order to quench any extracellular CA), all of the fluorescent signals

SSDI S0014-5793(96)00190-1

can be assumed to originate from within viable cells. Cell viability at the conclusion the experimental periods was found to be >90% in all cases as determined by trypan blue exclusion.

3. Results

3.1. Estimation of steady-state levels of LIP with CA

The CA method for detecting iron and analyzing LIP in K562 cells has been described previously [3]. It is based on the principle that CA loaded into cells binds a significant portion of the LIP, due to its high affinity constants for iron(II) and (III) which are similar to those of EDTA (10^{14} and 10^{24} M^{-1} respectively). The CA-bound iron is revealed by the addition of excess permeant chelator which scavenges the cytoplasmic iron, including the CA-bound fraction, and restores CA's fluorescence. The amplitude of the chelator-mediated rise in fluorescence is proportional to the amount of CA-bound iron, as illustrated in Fig. 1A. The cells used in this series of studies showed average LIP levels of 350 nM when grown in medium with 7% fetal calf serum [3].

3.2. Time dependence of LIP recovery following an acute iron load

We assessed the cells' capacity to restore LIP to basal levels after an acute rise in LIP (Fig. 1). Previously we have shown that iron(II) added as FAS rapidly enters K562 cells and accumulates in the chelatable pool [3,14]. The raw data showing the initial LIP rise in response to 20 μM FAS, and its subsequent decline, are shown in Fig. 1A. The kinetics of the recovery are shown in Fig. 1B. Immediately following a 10 min exposure of cells to 10, 20 and 40 μM FAS and resuspension in serum-containing medium, the respective LIP increased to 137 ± 10%, 154 ± 10% and 226 ± 46% of control (Table 1). Thus the magnitude of the initial rise in LIP was commensurate with the concentration of FAS applied. The return to control LIP levels following iron load was biphasic, comprising an initial (30 min) rapid recovery, succeeded by a relatively slower phase. The time required for LIP to decrease by 50% (i.e. $t_{1/2}$ of recovery from initial maximum to control level) was 27 min in cells treated with 10 and 20 μM FAS, and 55 min in cells treated with 40 μM FAS. However, only cells treated with 10 μM FAS fully recovered to control values within the 90 min time scale of this experiment.

Table 1
Half-times of LIP recovery from various iron load conditions[a]

Pretreatment[b]		Initial LIP[c] (% of control)	Recovery[d] $t_{1/2}$ (min)
FAS	10 μM	137 ± 10	27
	20 μM	154 ± 10	27
	40 μM	226 ± 46	55
H_2O_2	63 μM	117 ± 4	63
	125 μM	132 ± 5	88
	250 μM	149 ± 12	69
TBHP	100 μM	134 ± 6	65
ME	2.5 mM	127 ± 10	≤17
	5.0 mM	142 ± 2	≤17
FAC O/N		178 ± 5	73
FAC O/N & FAS		200 ± 10	63
(iron-free) O/N & FAS		164 ± 7	33
(iron-free) O/N & H_2O_2		140 ± 8	>90

[a]Values are from data in Figs. 1–3.
[b]All treatments were for 10 min at 37°C unless indicated as O/N (18 h).
[c]Measured immediately following the treatment and given as % of untreated, iron-replete control (± S.E.M., $n = 3$).
[d]Estimated time required for LIP to return half-way toward untreated iron-replete control levels (from Figs. 1–3).

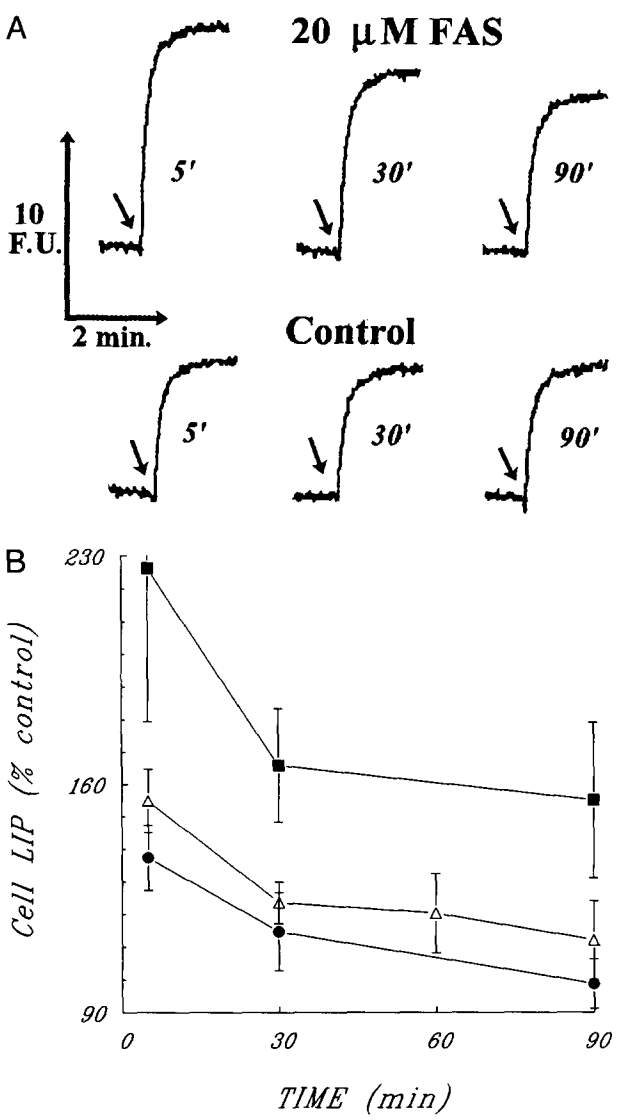

Fig. 1. Recovery of LIP following an acute iron load. A: K562 cells (iron-replete) were incubated in the absence (control) or presence of 20 μM FAS for 10 min at 37°C, centrifuged and resuspended in serum-free medium, and further incubated at 37°C. At the times indicated a sample of cells was removed, loaded with CA, and assayed for LIP as shown. The addition of 100 μM SIH is indicated by arrows. B: Cells were treated at FAS concentrations of 10 (●), 20 (△), and 40 μM (■) and LIP was monitored as in A. Results shown are relative to untreated control.

3.3. Recovery of LIP following a transient oxidative and reductive load in iron-replete and iron-starved cells

Oxidative as well as reductive stress caused a dose-dependent increase in LIP. Immediately after treating iron-replete cells (cultured for 18 h with 50 μg/ml human Tf) for 10 min at 37°C with 125 and 250 μM H_2O_2, and 100 μM *tert*-butylhydroperoxide (TBHP), LIP rose to 132 ± 5%, 149 ± 12 and 134 ± 6% of control respectively (Fig. 2A). This was followed by a gradual return to control values, with respective $t_{1/2}$s of recovery of 88, 69 and 65 min. Exposure to 2.5 and 5 mM mercaptoethanol (ME) also caused LIP to rise to 127 ± 10%

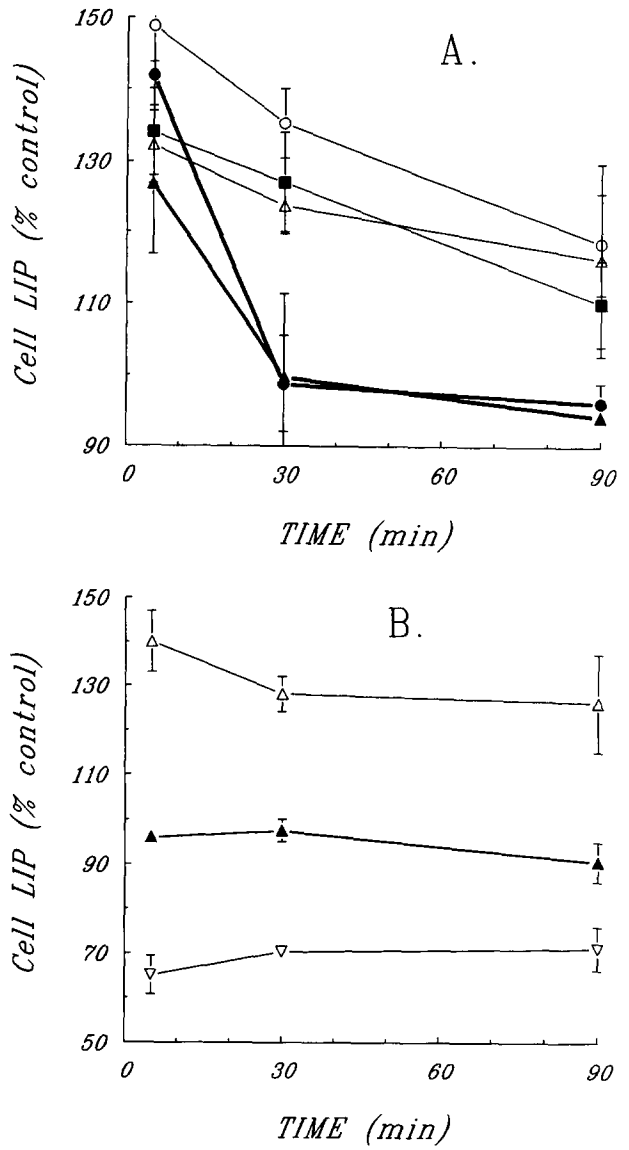

Fig. 2. Recovery of LIP following an acute oxidative and reductive treatment. A: Iron-replete K562 cells were exposed to 0.125 mM H_2O_2 (△), 0.25 mM H_2O_2 (○), 0.1 mM TBHP (■), 2.5 mM ME (▲) and 5.0 mM ME (●), for 10 min at 37°C. The cells were centrifuged and resuspended in serum-free medium and incubated at 37°C. At the times indicated a sample of cells was removed, loaded with CA, and LIP was assayed. B: Iron-starved cells (▽) were treated with 0.125 mM H_2O_2 (△) and 2.5 mM ME (▲), as above and similarly assayed. All results shown are relative to untreated, iron-replete control.

and 142 ± 2% of control (Fig. 2). However, following ME treatment, LIP rapidly (<30 min) returned to control levels.

Iron-starved cells, which had been cultured for 18 h in the absence of added iron sources, had a reduced initial LIP, amounting to 65 ± 4% of iron-replete control (Fig. 2B). Following exposure to 125 μM H_2O_2 and 2.5 mM ME their respective LIPs rose initially to 140 ± 7 and 96 ± 2% of the untreated iron-replete control. However, no significant recovery toward normal LIP levels was observed in these cells.

3.4. *Recovery of LIP following an acute iron load in iron-replete, starved and overloaded cells*

We assessed the influence of cellular iron status on the rate of LIP recovery from an acute iron load (Fig. 3). The initial LIP was elevated to 178 ± 5% in iron-overloaded cells and reduced to 74 ± 3% in iron-starved cells, relative to iron-replete ones. After a 10 min (acute) exposure to 20 μM FAS, the respective LIPs of iron-overloaded, starved and replete cells increased to 200 ± 10%, 164 ± 7% and 168±15% (all compared to untreated iron-replete control). The rates of recovery to iron-replete, untreated control values were essentially the same in iron-starved and replete cells, $t_{1/2}$s of both were 33 min. However, in iron-overloaded cells the kinetics of recovery from an acute iron load were superimposed on the kinetics of recovery from the long-term, 18 h iron overload. Yet, the $t_{1/2}$s of LIP recovery were roughly similar in both cases – 63 and 73 min respectively. Thus, K562 cells retain the capacity, albeit restricted, to readjust LIP after a chronic iron load.

The altered iron status of the cells cultured under different iron supply conditions was reflected in their relative ferritin content, as estimated by immunoblotting (Fig. 4). Iron-starved cells had ferritin levels below the level of detection, whereas iron-overloaded cells contained 5-fold more ferritin than iron-replete cells.

4. Discussion

The 'labile iron pool' (LIP) is an operational definition for cytosolic iron which is in ionic form, probably iron(II), is accessible to chelators, and is therefore presumed to be weakly bound. LIP is the iron form which is surmised to be sensed by IRPs. However, the dynamics of LIP-sensing mechanisms and their actual operation as modulators of iron sequestration

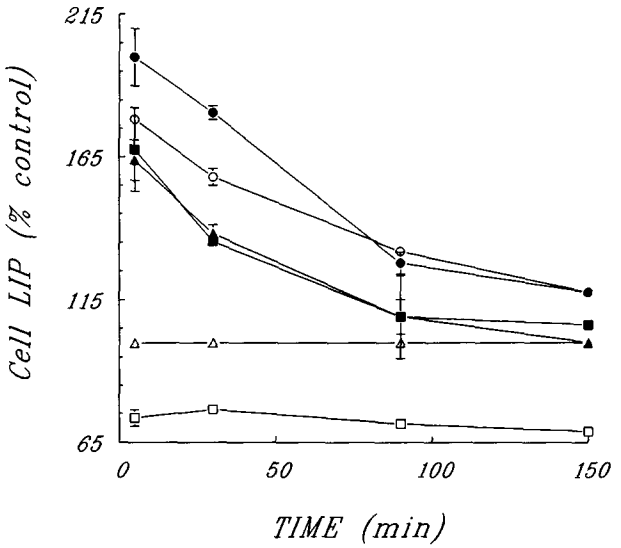

Fig. 3. Recovery of LIP in iron-loaded and iron-starved cells. K562 cells were cultured for 18 h in serum-free culture medium containing 1 mg/ml BSA with the following additions: human transferrin 50 μg/ml (iron-replete); 100 μM ferric ammonium citrate, FAC (iron-overloaded); no addition (iron-starved). The cells were centrifuged and resuspended in α-MEM-Hepes medium and divided into two portions, one of which was exposed to 20 μM FAS for 10 min at 37°C. The cells were then centrifuged and resuspended in serum-free culture medium and incubated at 37°C. At the times indicated samples of cells were removed, loaded with CA, and LIP was assayed. Iron-starved, untreated: □; iron-starved, FAS treated: ■; iron-replete, untreated (control): △; iron-replete, FAS treated: ▲; iron-overloaded, untreated: ○; iron-overloaded, FAS treated: ●. Results shown are relative to iron-replete, untreated control.

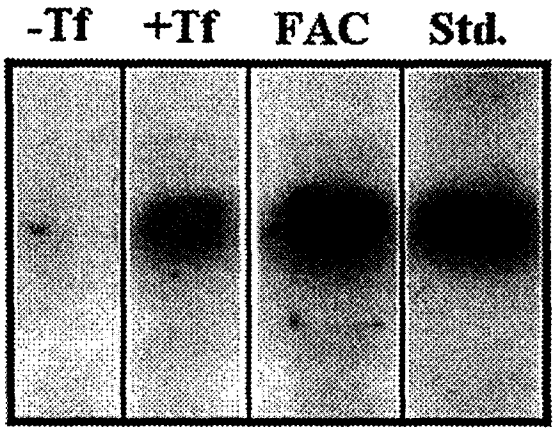

Fig. 4. Ferritin levels in iron-replete, overloaded and starved cells. Cells were cultured for 18 h with either 50 μg Tf/ml (+Tf'), or 100 μM FAC (FAC), or no iron source (−Tf). They were then centrifuged and solubilized with 0.25% Triton X-100. 40 μg of cell extract protein from each sample was separated by SDS-PAGE on 10–15% acrylamide gels. After electrotransfer, the blot was probed with a rabbit polyclonal antibody against human spleen ferritin, followed by horseradish peroxidase-conjugated sheep anti-rabbit IgG, and developed in enhanced chemiluminescence (ECL) reagent. Purified human ferritin was run as a standard (Std.).

systems in living mammalian cells have yet to be directly elucidated [2,11]. A major impediment has been the difficulty in temporally and spatially tracing the putative form of iron which is 'sensed' by cells, i.e. the LIP.

In this work we adopted a direct approach for determining cellular LIP, followed its dynamics and assessed the cellular capacity for maintaining steady levels of LIP. We used K562 cells because of the large amount of information available on iron metabolism in these cells [15,16]. We focused primarily on how variable iron supply conditions affect LIP, and assessed the cells' capacity to restore LIP to homeostatic levels following evoked changes in LIP. The studies are based on our previous demonstration that the fluorescent, iron-sensitive probe CA, in conjunction with membrane permeant chelators, can provide a valid measure of LIP [3].

4.1. Chronic iron overload

In K562 cells logarithmically growing in medium with 7% fetal calf serum, the steady state LIP estimated by this technique was 350 ± 100 nM, of which >80% was in the form of iron(II) [3]. After an 18 h incubation, LIP rose about 2-fold when the medium was supplemented with ionic iron(III), given as 100 μM FAC (Fig. 3). Changes in cell iron load are known to be accompanied by concomitant changes in ferritin levels in these and other cells (Fig. 4, also ref. [16]). Consistent with the function of ferritin as a major iron-sequestering moiety, chronic iron loads of ≤25 μM FAC produced little or no detectable rise in LIP (data not shown). However, 100–200 μM FAC caused an apparently uncontrollable expansion in the chelatable iron pool, which failed to be matched by the sequestration capacity of ferritin. This is consistent with the observations that ingress of iron salts into cells via non-Tf mediated uptake pathways, is not down-regulated by iron overload [17,18], and is even enhanced in some cell types [19].

4.2. Acute iron load

The rapid sequestration of iron from the chelatable pool is of particular importance in situations where cells are exposed to a sudden iron load. It is well established that oxidative damage caused by tissue ischemia/reperfusion can be significantly reduced by iron chelators. The basis of this preventive effect is believed to be the chelation of iron released from storage compartments, which in combination with the sudden oxidative burst following reperfusion, causes a rapid accumulation of oxidative radicals (reviewed in [21,22]). Iron-related (chelator-inhibitable) oxidative injury has also been observed in a variety of pathological situations: endothelial cells exposed to sources of heme [23], hearts undergoing hypothermia [24] or ischemia/reperfusion [25,26], erythrophagocytosing Kupffer cells [27], the CNS after mechanical trauma [28] and liver cells exposed to agents such as ethanol [29] and diquat [30]. Thus, an endogenous mechanism for rapid re-sequestration of excess cytoplasmic iron would minimize oxidative damage, similarly to the effect of externally added chelators. The efficiency of this mechanism could be an important determinant of the capacity of cells to recover from or resist potential damage from transient iron loads.

We simulated the above pathological conditions in the K562 cell model system. Restoration of LIP to resting levels, was markedly longer after an acute iron load of 40 μM FAS as compared to 10 or 20 μM FAS (Fig. 1). This would be expected if at high concentrations the iron load saturated and exceeded the limited absorption capacity of the cells. A short exposure of cells to oxidative (H_2O_2) or to a reductive (ME) stress also produced a dose-dependent rise in LIP (Fig. 2). In both cases this phenomenon might be attributed to reductive iron release from ferritin, as has been shown in vitro for superoxide radicals [31] and sulfhydryl reagents [20]. The restoration of the released iron to basal levels was notably slower in H_2O_2-treated and faster in ME-treated cells than in controls. Possibly, the iron sequestration mechanism, which might involve sulfhydryl groups, is inhibited by oxidative stress due to H_2O_2 and stimulated by the reducing agent ME. Alternatively, the two agents may release iron from different sources (not necessarily ferritin), with different affinities for iron, resulting in different rates of re-sequestration. We favor the latter alternative as it is consistent with the results obtained with iron-starved cells (Fig. 2B). In these cells the amount of iron released by 0.125 mM H_2O_2 was considerably greater than by 2.5 mM ME, while in iron-replete cells the amount released by both reagents was similar (Fig. 2A). Since iron-starved cells have severely depleted ferritin stores (Fig. 4), it is conceivable that H_2O_2 released iron from non-ferritin sources, while ME released iron from ferritin.

4.3. Cellular iron status and LIP homeostasis

We examined the influence of the cellular iron status on the rate of iron sequestration following an acute iron(II) load (Fig. 3). Cells which had been partially iron-depleted by 18 h culture in serum-free medium showed an accordingly depressed LIP level. Since their LIP returned to control levels at the same rate as iron-replete cells, it would appear that the sequestration system is not markedly affected by previous iron starvation. On the other hand, recovery of LIP is 2-fold slower after a long-term iron overload (18 h FAC) than after a brief (10 min FAS) iron load. If long-term iron overload is associated with oxidative stress, this observation would further support the idea that iron sequestration capacity may be influenced by the oxido-reductive state of the cells.

The present model cell system for studying LIP and cell iron sequestration mechanisms enables us to address various questions related to possible relationships between the rate of iron sequestration and cellular (i) ferritin levels and iron status, (ii) IRP activity, (iii) oxido-reductive status and (iv) injury by xenobiotics or hypoxia/reoxygenation. Application of these techniques to other cell types will hopefully permit comparative studies of the modes of handling of labile iron in cells from different tissues and genetic origins.

Acknowledgements: We wish to thank Hava Glickstein for excellent assistance and Dr. P. Ponka for kindly providing SIH. Supported in part by the Israel National Science Foundation and by NIH grant AI20342.

References

[1] Jacobs, A. (1977) Blood 50, 4331–4336.
[2] Crichton, R.R. and Ward, R.J. in Iron and Human Diseases, R.B. Laufer, Ed., 1992, pp. 23–76. CRC Press, Boca Raton, FL.
[3] Breuer, W., Epsztejn, S. and Cabantchik, Z.I. (1995) J. Biol. Chem. 270, 24209–24215.
[4] Hershko, C. and Weatherall, D.J. (1988) CRC Crit. Rev. Clin. Lab. Sci. 26, 303–345.
[5] Rothman, R.J., Serroni, A. and Farber, J.L. (1992) Mol. Pharmacol. 42, 703–710.
[6] Ponka, P., Grady, R.W., Wilczynska, A. and Schulman, H.M. (1984) Biochim. Biophys. Acta 802, 477–489.
[7] Baker, E., Richardson, D., Gross, S. and Ponka, P. (1990) Hepatology 15, 492–501.
[8] Yegorov, D.Y., Kozlov, A.V., Azizova, O.A. and Vladimirov, Y.A. (1993) Free Radical Biol. Med. 15, 565–574.
[9] Halliwell, B. and Gutteridge, J.M.C. (1990) Methods Enzymol. 186, 1–85.
[10] Zhan, H., Gupta, R.K., Weaver, J. and Pollack, S. (1990) Eur. J. Haematol. 44, 124–130.
[11] Klausner, R.D., Rouault, T.A. and Harford, J.B. (1993) Cell 72, 19–28.
[12] Constable, A., Quick, S., Gray, N.K. and Hentze, M.W. (1992) Proc. Natl. Acad. Sci. USA 89, 4554–4558.
[13] Sakaida, I., Kyle, M.E. and Farber, J.L. (1990) Mol. Pharmacol. 37, 435–442.
[14] Breuer, W., Epsztejn, S., Millgram, P. and Cabantchik, Z.I. (1995) Am. J. Physiol. 268, C1354–1361.
[15] Roberts, S. and Bomford, A. (1988) J. Biol. Chem. 263, 19181–19187.
[16] Toth, I., Rogers, J.T., McPhee, J.A., Elliott, S.M., Abramson, S.L. and Bridges, K.R. (1995) J. Biol. Chem. 270, 2846–2852.
[17] Wright, T.L., Brissot, P., Ma, W.-L. and Weisiger, R. (1986) J. Biol. Chem. 261, 10909–10914.
[18] Inman, R.S. and Wessling-Resnick, M. (1993) J. Biol. Chem. 8521–8528.
[19] Parkes, J.G., Randell, E.W., Olivieri, N.F. and Templeton, D.M. (1995) Biochim. Biophys. Acta 1243, 373–380.
[20] Funk, F., Lenders, J.-P., Crichton, R.R. and Schneider, W. (1985) Eur. J. Biochem. 152, 167–172.
[21] Sussman, M.S. and Bulkley, G.B. (1990) Methods Enzymol. 186, 711–722.
[22] Ryan, T.R. and Aust, S.D. (1992) Crit. Rev. Toxicol. 22, 119–141.
[23] Balla, J., Jacob, H.S., Balla, G., Nath, K., Eaton, J.W. and Vercellotti, G.M. (1993) Proc. Acad. Natl. Sci. USA 90, 9285–9289.
[24] Magni, F., Panduri, G. and Paolocci, N. (1994) Free Radical Biol. Med. 16, 465–476.
[25] Voogd, A., Sluiter, W. and Koster, J.F. (1994) Free Radical Biol. Med. 16, 453–458.
[26] Chevion, M., Jiang, Y., Har-El, R., Berenshtein, E., Uretzky, G. and Kitrossky, N. (1993) Proc. Natl. Acad. Sci. USA 90, 1102–1106.
[27] Kondo, H., Saita, K., Grasso, J.P. and Aisen, P. (1988) Hepatology 8, 32–38.
[28] Halliwell, B. and Gutteridge, J.M.C. (1985) Trends Neurosci. 8, 22–25.
[29] Shaw, S. (1989) J. Free Radical Biol. Med. 7, 541–546.
[30] Reif, D.W., Beales, I.L.P., Thomas, C.E. and Aust, S.D. (1988) Toxicol. Appl. Pharm. 93, 506–510.
[31] Thomas, C.E. and Aust, S.D. (1986) J. Biol. Chem. 261, 13064–13070.
[32] Lichtenfels, R., Biddison, W.E., Schulz, H., Vogt, A.B. and Martin, R. (1994) J. Immunol. Methods 172, 227–239.