Free Rad. Res., Vol. 29, pp. 103–114
Reprints available directly from the publisher
Photocopying permitted by license only

Endogenous Ferritin Protects Cells with Iron-Laden Lysosomes against Oxidative Stress

BRETT GARNER[a], KARIN ROBERG[b] and ULF T. BRUNK[b,*]

[a]*Department of Chemistry, University of Wollongong, Wollongong NSW 2522, Australia;* [b]*Division of Pathology II, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden*

Accepted by Prof. B. Halliwell

(Received 2 February 1998; In revised form 16 March 1998)

Previous studies have shown that a variety of mammalian cell types, including macrophages, contain small amounts of redox-active iron in their lysosomes. Increases in the level of this iron pool predispose the cell to oxidative stress. Limiting the availability of intralysosomal redox-active iron could therefore represent potential cytoprotection for cells under oxidative stress.

In the present study we have shown that an initial 6 h exposure of J774 macrophages to 30 μM iron, added to the culture medium as $FeCl_3$, increased the lysosomal iron content and their sensitivity to H_2O_2-induced (0.25 mM for 30 min) oxidative stress. Over time (24–72 h), however, the cells were desensitized to the cytotoxic effects of H_2O_2; most likely as a consequence of both lysosomal iron exocytosis and of ferritin synthesis (demonstrated by atomic absorption spectrophotometry, autometallography, and immunohistochemistry). When the cells were exposed to a second dose of iron, their lysosomal content of iron increased again but the cells became no further sensitized to the cytotoxic effects of H_2O_2. Using the lysosomotropic weak base, acridine orange, we demonstrated that after the second exposure to iron and H_2O_2, lysosomes remained intact and were no different from control cells which were exposed to H_2O_2 but not iron.

These data suggest that the initial induction of ferritin synthesis leads to enrichment of lysosomes with ferritin via autophagocytosis. This limits the redox-availability of intralysosomal iron and, in turn, decreases the cells' sensitivity to oxidative stress. These *in vitro* observations could also explain why cells under pathological conditions, such as haemochromatosis, are apparently able to withstand high iron concentrations for some time *in vivo*.

Keywords: Autophagocytosis, ferritin, iron, lysosomes, oxidative stress

INTRODUCTION

Iron overload has been shown to be cytotoxic *in vitro* and is also associated with tissue damage in various pathological states *in vivo*, e.g. hepatic cirrhosis, primary hepatic carcinoma, atherosclerosis, and diabetes.[1–4] The lysosomal compartment is an important storage site for iron which may be taken up from different sources including from iron salts and chelates, damaged erythrocytes, and through normal autophagocytic activity.[5–10] Lysosomes are generally

*Corresponding author. Tel.: +46-(0)13-22 15 15. Fax: +46-(0)13-22 15 29. E-mail: ulf.brunk@pat.liu.se.

considered stable organelles and it has been suggested that degradation of metalloproteins (such as ferritin) within membrane-encapsulated secondary lysosomes helps to prevent the uncontrolled release of iron within the cell and thereby limit its cytotoxicty.[11] Apart from a few exceptional cases, lysosomes are not believed to induce cellular damage by leaking their hydrolytic enzymes to the cytosol. However, we have recently questioned this and shown that the cytotoxic effects of oxidative stress are often a consequence of intralysosomal iron-catalyzed oxidative reactions with ensuing membrane damage and leakage of hydrolytic lysosomal enzymes to the cytosol.[5,6,12] This chain of events may result in either apoptotic or necrotic cell death.[13] Furthermore, there is some evidence that intralysosomal iron accumulation damages lysosomal membranes *in vivo* as they are more fragile in liver samples taken from iron-loaded human subjects and experimental animals.[1,14] It is plausible that this increased fragility is due to lysosomal membrane oxidation.[1]

We hypothesize that cellular sensitivity to oxidative stress is mainly a function of: (i) their capacity to degrade H_2O_2, preventing its diffusion into the lysosomal compartment; and (ii) the intralysosomal concentration of redox-active iron. The redox availability of iron can be minimized by the lysosomal presence of endocytosed iron-chelators, such as deferrioxamine.[5,12] We recently showed that endocytosed ferritin was also targeted to the lysosomal compartment where it was able to exert a cytoprotective role; at least partially by virtue of its ability to enhance lysosomal stability under conditions of iron/H_2O_2-induced oxidative stress.[5]

In the present study we have investigated the efficacy of endogenously synthesized ferritin as a cytoprotective agent in cells under oxidative stress. The possibility that such endogenous ferritin can protect iron-loaded lysosomes is also addressed. We show that exposure of macrophage-like J-774 cells to hydrated ferric-phosphate complexes, which are actively endocytosed and directed to the acidic vacuolar compartment, initially enhanced their sensitivity to oxidative stress and destabilized their lysosomes. Later, in parallel with the relocalization of lysosomal iron to the cytosol and increased ferritin formation, the enhanced sensitivity disappeared. Finally, the cells withstood a second endocytotic uptake of the iron-complex without any increased sensitivity to oxidative stress. We interpret the findings to be an effect of random autophagocytosis enriching the lysosomes with iron-unsaturated ferritin. The results suggest that iron occurring intralysosomally as a complex with ferritin does not efficiently catalyze oxidative reactions and may remain undegraded for some time.

MATERIALS AND METHODS

Chemicals

Glutamine, penicillin-G and streptomycin were from Flow (Rickmansworth, UK). Ham's F-10 medium and dialyzed fetal calf-serum were from GIBCO (Paisley, UK), while uranyl acetate and lead nitrate were from Merck (Darmstadt, Germany), and paraformaldehyde, silver-lactate and Epon-812 from Fluka AG (Buchs, Switzerland). Glutaraldehyde was from Bio-Rad (Cambridge, MA, USA), osmium tetroxide from Johnson Matthey Chemicals (Roystone, UK), and goat anti-rabbit IgG-Texas Red conjugate from Vector Laboratories (Burlingame, USA). Rabbit anti-human ferritin polyclonal antibody was from DAKO (Carpeinteria, USA), ammoniumsulfide and hydroquinone were from BDH Ltd (Poole, UK) and saponin from Sigma (St. Louise, MO, USA). All other chemicals were of analytical grade and obtained from standard sources.

Cells and Culture Conditions

J-774 cells (an established mouse histiocytic lymphoma cell line) were routinely grown under humidified air with 5% CO_2 at 37°C in 75 ml

Costar (Cambridge, MA, USA) plastic culture flasks in Ham's F-10 medium supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS). Cells were detached for subcultivation once a week (1 : 2 split) by gentle scraping with a rubber policeman.

For experiments, cells were seeded into 35 mm Costar plastic petri dishes with or without glass coverslips (22 mm in diameter) at a density of 1.8×10^5 cells/dish in complete growth medium (2 ml/dish) and cultured for 24 h before experiments were initiated.

Iron Exposure

Cells, grown in 35 mm plastic dishes, with or without coverslips, were exposed to 30 μM ferric iron in complete growth medium for 6 h under standard culture conditions. A stock solution of $FeCl_3$ was added directly to the medium prior to loading cells with Fe. Unless prepared under acidic conditions, $FeCl_3$ will rapidly hydrolyze and polymerize. This is predicted to form insoluble, hydrated iron–phosphate complexes which are taken up through endocytosis by the cells. In agreement with this, added iron was initially present only in the acidic vacuolar compartment of the macrophages. Following the iron-exposure, cells were returned to standard culture conditions for another 24, 48 or 72 h and then again exposed to the iron-complex for 6 h, rinsed and returned to normal culture conditions for another hour (to make sure no iron would be attached to the cell surfaces).

Oxidative Stress

The control and iron-treated cells were exposed to oxidative stress using a bolus dose of 0.25 mM H_2O_2 in PBS at 37°C for 30 min. Cells intended for estimation of viability were then returned to ordinary culture conditions for another 16 h (see below), while cells to be used for measurement of lysosomal stability were initially exposed to the lysosomotropic fluorochrome acridine orange, then to oxidative stress, rinsed and directly studied by static fluorometry as described below.

Determination of Cell Viability after Exposure to Oxidative Stress

Cell viability was determined by the delayed trypan blue dye exclusion test.[6] The control and iron-exposed cells were initially exposed to oxidative stress as described above, then returned to ordinary culture conditions for another 16 h and finally stained with trypan blue (0.1% in PBS for 2 min at 22°C). In each culture, the number of stained (dead) and unstained (living) cells were counted, using an inverted microscope, in ten random fields of vision at low magnification (×250).

Estimation of Cellular Ferritin Levels by Immunofluorescence

Cells, grown on coverslips, were exposed to 30 μM ferric iron (see above) for 6 h under otherwise ordinary culture conditions. They were then rinsed and returned to normal culture medium. After another 24, 48 or 72 h they were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, rinsed in PBS and exposed to 0.1% saponin and 5% FCS in PBS for 20 min at room temperature. The coverslips were then placed in a humidifier, 100 μl anti-ferritin (1 : 100 in PBS with 0.1% saponin and 5% FCS) was added to each coverslip, and the cells were incubated at 4°C overnight. They were then rinsed (2 × 5 min) in PBS with 0.1% saponin and 5% FCS, after which 100 μl anti-IgG Texas Red conjugate (1 : 200 in PBS with 0.1% saponin and 5% FCS) was added, and the cells were incubated for another 60 min at room temperature. Following a rinse in PBS, the coverslips were mounted in a drop of PBS on excavated microculture slides. The intensities of red fluorescence from each of

100 cells/coverslip were measured in a computer-based static cytofluorometer system, based on a Nikon microphote SA, using green exciting light and a red barrier filter (G-1B, DM 580 Nikon filter cube).

Determination of Total Cellular Iron

The total amount of cellular iron was analyzed using a Polarized Zeeman atomic absorption spectrophotometer Z 8270 (Hitachi, Tokyo, Japan) equipped with an iron lamp (243.3 nm). Cells from each dish were lysed in 500 µl distilled water (MilliQ, Millipore) and stored at −70°C until assayed. Before measurement the suspension was further diluted five times in distilled water. Five dishes were used for each point of time. Distilled water was used as a blank. Iron was related to the protein content that was determined by the Lowry method.

Autometallography for Heavy Metal Cytochemistry

The procedure for heavy metal cytochemistry was as previously described.[15] Briefly, cells grown on coverslips were rinsed in PBS (22°C) prior to fixation with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer with 0.1 M sucrose (pH 7.2; effective osmotic pressure 300 mOsm) for 2 h at 22°C. The fixation was followed by short rinses (×5) in glass-distilled water at 22°C. Cells were then sulfidated at pH ≈9 in 1% (w/v) ammonium sulfide in 70% (v/v) ethanol for 15 min. Following careful rinsing in running, glass-distilled water for 10 min at 22°C, development was performed according to Danscher[16,17] using a physical, colloid-protected developer containing Ag-lactate. The reaction was performed in the dark at 26°C for 20, 25 and 30 min. Following dehydration in a graded series of ethanol solutions and mounting in Canada balsam,[15] the cells were examined and photographed using Kodak Tri-X pro 400 ASA film.

Transmission Electron Microscopy

Control and iron-exposed cells were prepared for TEM as previously described.[18] Briefly, they were fixed *in situ* in the plastic dishes by the addition of a buffered glutaraldehyde-based fixative and post-fixed in osmium tetroxide. Dehydration, staining en bloc with uranyl acetate, dehydration and embedding in Epon-812 was also performed in the culture dishes. Thin sections of the cured blocks were cut with a diamond knife, stained with lead-citrate, examined, and photographed in a JEOL 2000-EX electron microscope (Tokyo, Japan) at 100 kV.

Degradation of Hydrogen Peroxide

Cells (1.8×10^5 cells/dish) were exposed to 0.5 mM H_2O_2 in PBS (2 ml/dish). The degradation of H_2O_2 was measured by horseradish-peroxidase-mediated H_2O_2-dependent pHPA oxidation[19] and followed for up to 60 min. Fluorescence intensity was read at λ_{ex} 315 nm and λ_{em} 410 nm with an RF-540 spectrofluorometer (Shimadzu, Japan) connected to a DR-3 Data recorder.

Lysosomal Membrane Stability Assay

Control and iron-treated cells, growing on circular coverslips, were exposed to acridine orange in complete medium as described before.[5] Following exposure to oxidative stress the coverslips were directly inverted over microculture glasses containing a drop of PBS and studied by static cytofluorometry using green excitation and a red barrier filter in the above Nikon equipment (G-1B, DM 580 Nikon filter cube).

The lysosomotropic weak base, acridine orange, accumulates in acidic compartments and in a concentrated form it has a monochromatic red fluorescence when activated with green light (it has a concentration-dependent metachromatic red or green fluorescence when activated with blue light). The amount of red fluorescence

per cell reflects remaining lysosomes with intact proton gradients and high concentrations of acridine orange.

Statistical Analysis

Statistical significance was determined by using the unpaired Student's *t*-test. A *p*-value < 0.05 (*) was considered significant. $p < 0.01$ (**); $p < 0.001$ (***).

RESULTS

Cell Viability after Exposure to Oxidative Stress

It has been previously shown that cells exposed to iron become sensitized to H_2O_2-induced oxidative stress[6,20,21] and that an important underlying mechanism for this is due to lysosomal membrane damage caused by free radicals.[6,22] In the present study we initially investigated the duration of iron-sensitization and whether the cells previously exposed to iron acquired resistance to subsequent oxidative stress.

Control cells and cells treated with iron either once or twice were subjected to oxidative stress by exposure to a bolus dose of 0.25 mM H_2O_2 in PBS for 30 min. They were then rinsed in PBS and returned to ordinary culture conditions for another 16 h and finally stained with trypan blue. In previous experiments it was found that a delay of 16 h between the stress and the staining gave an accurate account of cell viability without causing any significant detachment of cells.[6] Figure 1 shows that cells exposed to iron and then directly to oxidative stress were more sensitive than control cells not exposed to iron. After the iron-exposure, prolonged periods under normal culture conditions diminished this sensitivity in such a way that iron-loaded cells were restored to the same sensitivity as the control cells (not iron loaded) after 48 or 72 h (data not shown). A second 6 h period of iron-exposure, after an interval of 24 h under normal culture conditions, did not further increase the sensitivity to oxidative stress (Figure 1). When the interval before the second iron exposure was prolonged to 48 or 72 h the cells were insensitive and behaved as the control cells not exposed to iron at all (Figure 1).

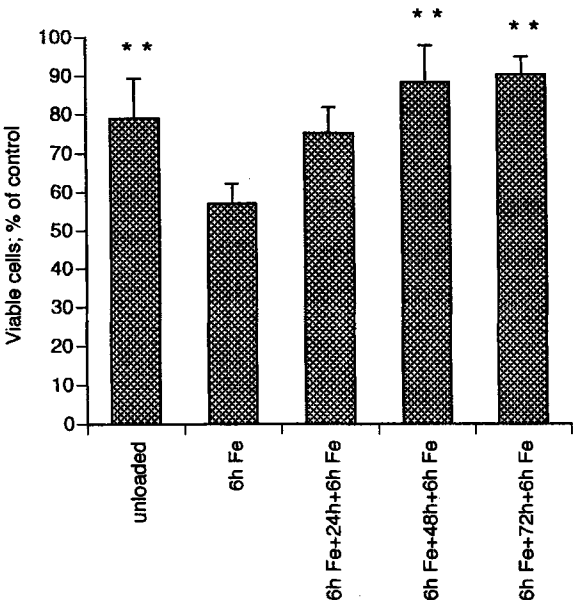

FIGURE 1 Viable cells, as assayed by the delayed trypan blue vital staining technique, following exposure to 0.25 mM H_2O_2 in PBS for 30 min at 37°C. Cells exposed to the iron-complex for 6 h and then to H_2O_2 showed about 60% viability which was significantly less than for cells not exposed to iron (unloaded), or exposed to iron for 6 h and then kept under normal culture conditions for another 48 or 72 h. Bars show Means ± S.D.; $n = 3$–5.

Several possible explanations could account for the desensitization of the cells to oxidative stress. Firstly, the iron which was present may have become chelated by ferritin and therefore remain but in a less redox active state; secondly, the cells could have released some of their iron back to the surrounding medium and thereby also depleted their intralysosomal stores; or thirdly, the cells may have increased their capacity to degrade H_2O_2 after iron exposure and thereby limited the occurrence of Fenton-type reactions. These possibilities were therefore addressed.

Cellular Amounts of Ferritin Shown by Immunofluorescence

As was predicted, iron exposure increased intracellular ferritin levels. Figure 2 shows that after a 6 h exposure to iron, ferritin synthesis was stimulated and over the subsequent 72 h, its levels had doubled. Previous studies from our group and others have shown that cytosolic ferritin (and exogenously added apoferritin) enters the lysosomal compartment where it remains relatively intact before being converted to haemosiderin.[5,23,24]

Total Cellular Iron

The initial 6 h exposure to iron increased total cellular iron levels about ten-fold (Figure 3). When returned to ordinary culture conditions a time-dependent decrease of total cellular iron was observed, resulting in a loss of ≈50% after 72 h. However, the values were still approximately five times higher than those of the control cells. A second 6 h exposure to iron resulted in a further pronounced increase in total iron, demonstrating an undiminished cellular capacity for endocytic uptake of the iron–phosphate complex.

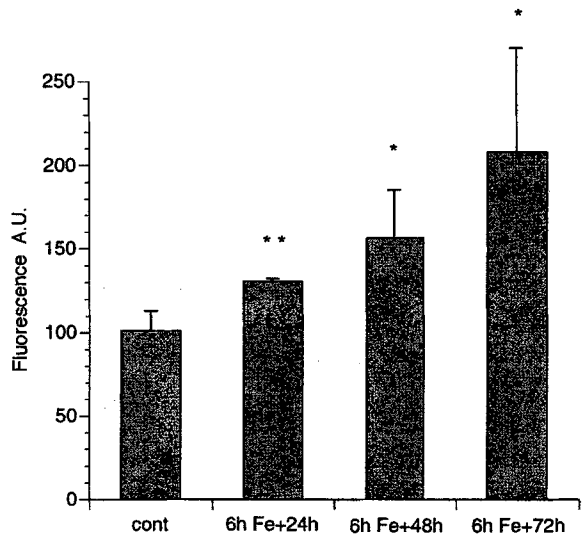

FIGURE 2 Effect on the cellular content of ferritin by exposure to the iron-complex for 6 h, followed by further culture at normal conditions for 24, 48, and 72 h. Ferritin was assayed by immonocytochemistry using a Texas-red labelled secondary antibody and static cytofluorometry as described in the Materials and Methods section. Bars show Means ± S.D.; $n = 3$–5.

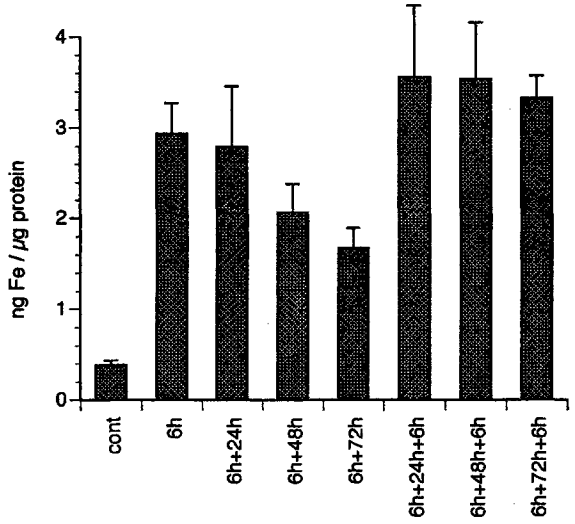

FIGURE 3 Total cellular content of iron, as assayed by atomic absorption spectrophotometry. Note the dramatic increase of cellular iron-content as a function of the exposure to the iron-complex for 6 h and the later decline in iron concentration. This decline is considered to be a result of cell division and lysosomal exocytosis of the iron-complex. Also note that the second exposure to the iron-complex results in a new increase of iron content of the same magnitude as the initial iron-exposure. Bars represent Means ± S.D.; $n = 5$.

Redox-Active Lysosomal Heavy Metal (iron) Cytochemistry

The silver sulfide method (SSM) is an extremely sensitive cytochemical method. It may be used for the demonstration of a variety of heavy metals, including iron, in toxicological studies.[15–17] Since in most normal cells metals other than iron are present in small amounts, the technique in practice may be used for demonstration of catalytically active cellular iron that can be converted into the iron sulfide. This implies that iron which is stably bound within metalloproteins is not readily demonstrable.[15] The SSM is a catalytic reaction, and the precipitation of silver is time-dependent. The lower the cellular amounts of iron sulfide, the longer the period of development is required. After exposure to the

iron-complex for 6 h the cells had endocytosed a considerable amount of iron and the development time had to be kept short (20 min) in order to avoid heavy over-staining. The low normal amount of lysosomal iron that is routinely observed[12,15] requires a longer development period than 20 min to be visualized and thus the control cells shown in Figure 4(A)

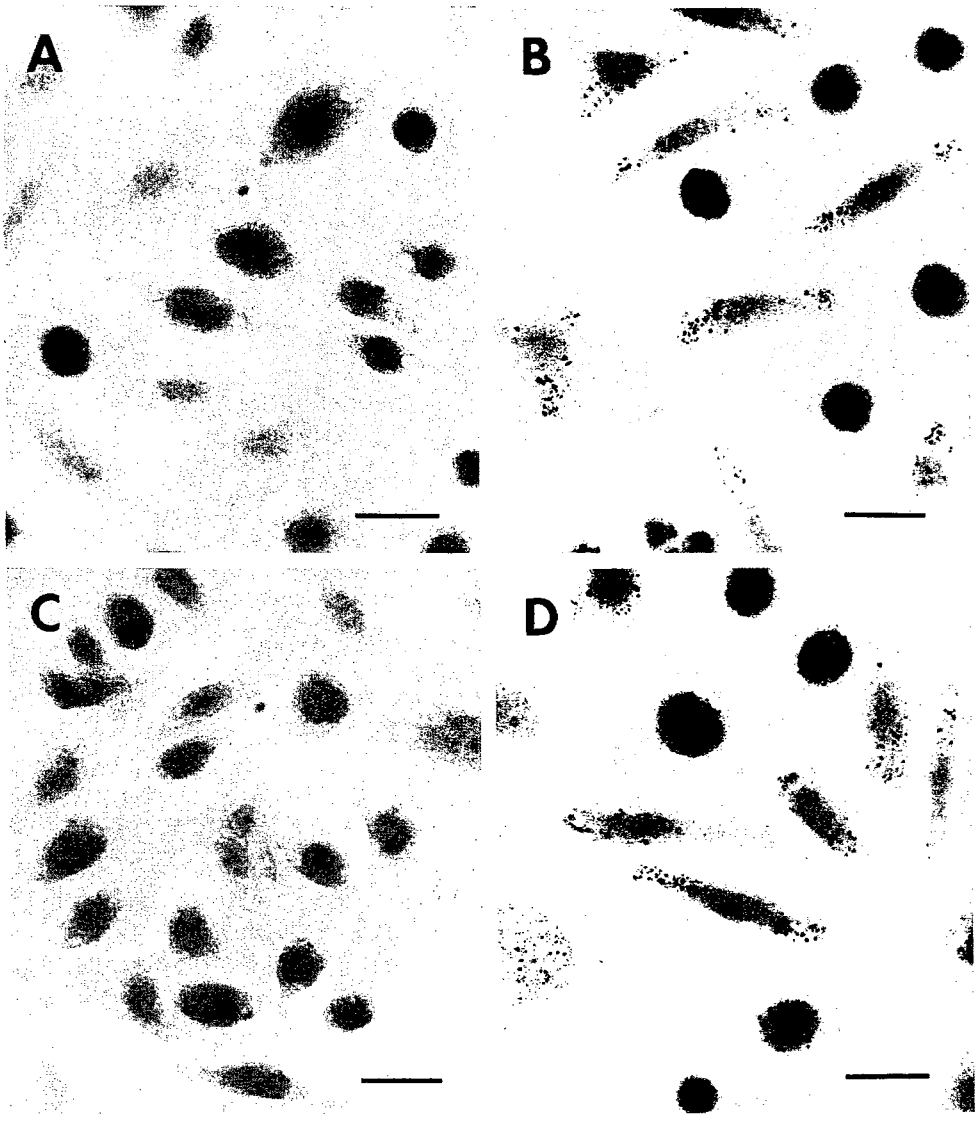

FIGURE 4 Cytochemical demonstration of iron by autometallography (SSM). Control cells (A) show no silver precipitates since the time used for physical development was 20 min which is not enough to allow demonstration of the normally occurring lysosomal low-molecular-weight iron. Cells exposed to the iron-complex for 6 h and then assayed (B) show numerous granules of a size and distribution which is typical for lysosomes. In (C) cells are shown which initially were exposed to the iron-complex for 6 h and then kept under normal culture conditions for another 72 h. Note the absence of reaction product indicating that the endocytosed iron was subsequently exocytosed and/or transported to the cytosol and bound in ferritin in a stable complex where it cannot be demonstrated with the SSM. Cells initially exposed to the iron-complex and then kept for 72 h under normal conditions and again exposed to the complex (D) are again capable of endocytosing iron into their acidic vacuolar apparatus. Compare with Figure 3. Bars = 20 μm.

show no lysosomal silver precipitation, while cells exposed to the iron-complex for 6 h show a distinct granularity of a lysosomal size and pattern. Following another 72 h under ordinary culture conditions, the lysosomal silver-precipitation pattern was gone, suggesting that some iron had been exocytosed from the acidic vacuome, while the remaining iron was chelated by newly synthesized ferritin which was delivered to lysosomes or had been transported into the cytosol and bound in ferritin in such a way that it was no longer available for the sulfide ions during the initial part of the SSM-technique. Following the second exposure to the iron-complex, the lysosomal pattern reappeared showing another uptake of iron into the acidic vacuolar apparatus. Bearing in mind the additional ferritin which was detected in these cells after 72 h (Figure 2), it seems plausible that the newly endocytosed iron was initially only loosely chelated by ferritin, although in such a way as to limit its availability for Fenton-type reactions but, at least partly, detectable by the SSM which is only a semi-quantitative technique.[15]

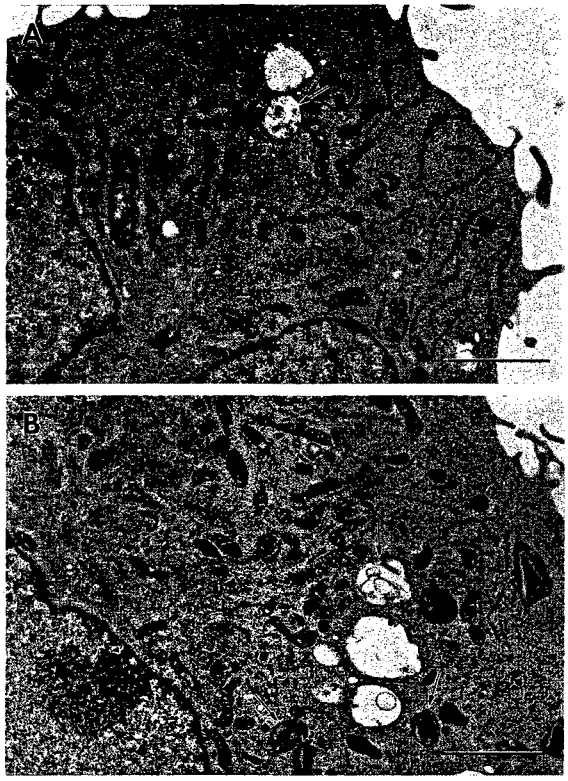

FIGURE 5 Fine structure of control cells (A) and cells exposed to the iron-complex for 6 h and then returned to normal culture conditions for another 72 h (B). Note slight increase in autophagocytotic activity as indicated by the arrowed figures in the iron-exposed cells, but otherwise identical morphology. Bars = 2 μm.

Transmission Electron Microscopy

As illustrated in Figure 5(A) and (B), exposure of the cells to iron resulted in slightly enhanced autophagocytosis as is demonstrated by the appearance of more autophagocytotic figures (arrowed). This is particularly evident in the cells initially exposed to iron-complex for 6 h and then kept under normal culture conditions for another 72 h. Otherwise, exposure to iron did not induce any significant alteration of the fine cellular morphology.

Degradation of Hydrogen Peroxide

Taken together, the results above suggested that the initial exposure of cells to iron, stimulates enough ferritin synthesis to enable rapid chelation of any additional iron which is added to the lysosomal compartment during the second exposure. This would limit the chances of intralysosomal free radical production under conditions of H_2O_2-induced oxidative stress. Alternatively, it was possible that the cells may have been better able to degrade H_2O_2 after iron treatment. Figure 6 shows, however, that there was no difference between the control and iron-exposed cells with respect to their capacity to degrade H_2O_2. In agreement with earlier work, it therefore appears that macrophage peroxide-degrading systems (i.e., catalase, GSH peroxidase) are not upregulated by iron exposure.[25]

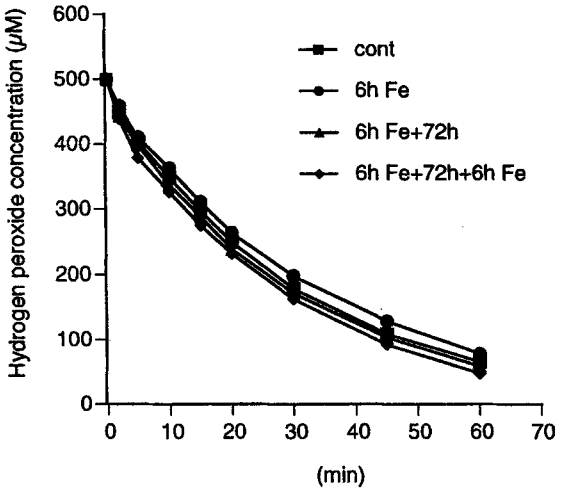

FIGURE 6 Degradation of time H_2O_2 by control cells, cells exposed to the iron-complex for 6 h, and cells exposed to iron for 6 h and then kept under normal culture conditions for another 72 h followed, or not, by a second iron-exposure. Note the same H_2O_2-degrading capacity for all four groups. The curves shown are from one typical experiment representative of four.

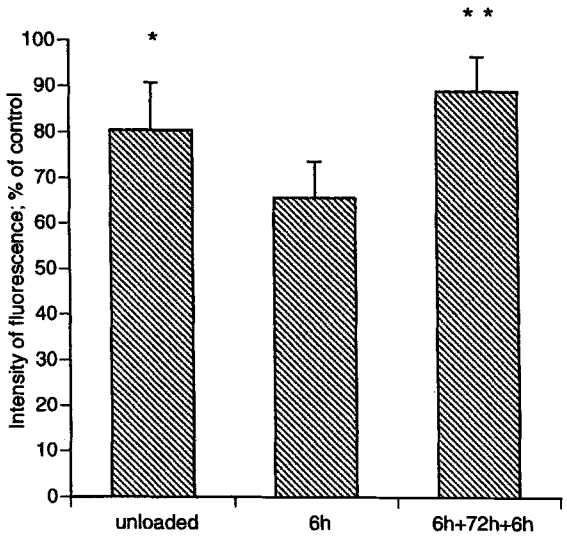

FIGURE 7 Lysosomal stability assay. Cells were exposed to the weak base acridine orange and then to oxidative stress as described in the Materials and Methods section. Lysosomal red fluorescence, indicating retention of the lysosomotropic drug was then measured by static fluorometry. Cells exposed to the iron-complex for 6 h showed significantly fewer intact lysosomes than unexposed control cells or cells initially exposed to the iron-complex, then kept under normal culture conditions for 72 h and, finally, again exposed to the iron-complex for another 6 h. Bars show Means ± S.D.; $n = 3$.

Lysosomal Membrane Stability

Finally, we investigated the stability of lysosomes after exposure to H_2O_2-induced oxidative stress and under conditions of iron loading. Confirming our previous studies,[5,6,12] the retention of acridine orange in a concentrated form (indicating normal lysosomes with low pH) declined significantly more in cells exposed to iron for 6 h than in control cells which were not exposed to iron (Figure 7). However, a second exposure to iron following a period of culture under normal conditions for 72 h resulted in retained lysosomal stability of the same magnitude as in the control cells (Figure 7).

DISCUSSION

Ferric iron, present in the growth medium as a hydrated phosphate-complex[26] is taken up by cultured cells as a result of endocytosis and accumulates intralysosomally, where it can be cytochemically demonstrated by autometallography, as shown here and in previous studies.[15] It is predicted that the acidic and reducing intralysosomal milieu converts iron to its ferrous form (e.g., the amino acid cysteine occurs intralysosomally in high concentrations[27]). In the presence of H_2O_2, ferrous iron in a redox-active form is a potent catalyst for the formation of the very aggressive hydroxyl radical ($HO^{\bullet}$).[3] In a state of oxidative stress, increased amounts of cytosolic H_2O_2 diffuse into the acidic vacuolar apparatus where iron-rich lysosomes would constitute a severe danger to the integrity of the cell, unless the iron is chelated in a non-redox-active form. Interestingly, recent studies have identified a transient ($t_{1/2} < 30$ min) labile iron pool that appears in the endosomes of human erythroleukemia K562 cells after endocytosis of transferrin.[28] These cells became sensitized to peroxide-induced oxidative stress after transferrin endocytosis and it was suggested that this damage may be mediated through released iron

binding to the lipid component of cell membranes.[28] It could be that endosomal damage contributes to the cytotoxicity of peroxides under these circumstances.

As suggested here and in earlier studies,[6,9,12] intralysosomal iron-catalyzed oxidative reactions may be particularly hazardous to the cell as the stability of lysosomes can be jeopardized, with relocation to the cytosol of hydrolytic enzymes and ensuing cellular damage or even death. The stability of lysosomes in relation to cellular degeneration has been a matter of dispute ever since their discovery by Christian de Duve, who, in order to emphasize their potential harmfulness to the living cell, somewhat provocatively nicknamed them "suicidal bags".[29] Current opinion amongst most authors, however, is that lysosomes are rather stable organelles which break down only late during cellular degeneration and do not initiate damage by releasing their numerous hydrolytic enzymes into the cytosol.

In opposition to the latter notion, and in support of de Duve's original hypothesis of lysosomes as suicidal bags, we advocate the idea that secondary lysosomes, due to their normal content of redox-active low-molecular-weight iron in small amounts, rather may be quite vulnerable, especially to oxidative stress.[5,6] The occurrence of such intralysosomal iron, which may constitute the largest part of the pool of cellular low-molecular-weight iron, would be a consequence of normal autophagocytotic degradation of a variety of metalloproteins, e.g., mitochondrial and endoplasmic cytochromes. As a consequence, cellular resistance to oxidative stress closely parallels lysosomal stability under oxidative stress conditions.[5,6]

Engulfed lysosomal iron-complexes would eventually be degraded and the iron transported to the cytosol, where formation of apoferritin would be initiated through binding to iron-binding proteins which, in turn, regulate the iron-responsive elements of ferritin mRNA. The synthesis of apoferritin permits the storage of iron in its ferric state[30] and in a mainly non-redox-active form.[31] This transformation process is visualized by the outcome of the cytochemical demonstration of iron by using SSM. Just after uptake, such iron is present in lysosomes, while restoration of normal culture conditions for 48–72 h results in its disappearance. Judged in the light of the findings of total cellular iron (i.e., that even after 72 h, there is still a five-fold increase in total iron present), and the increased amounts of ferritin present, it is obvious that the low-molecular-weight lysosomal iron has been converted into a form which is not demonstrable with the cytochemical (SSM) technique, our interpretation being that iron is integrated in a metalloprotein complex, such as ferritin.

Like other cytosolic components, ferritin is normally autophagocytosed in a process that is random, and at a rate that is proportional to its cytosolic presence.[32] In cells with large amounts of cytosolic ferritin, their lysosomes would contain comparatively large amounts of ferritin, most of which would be unsaturated with iron. Low-molecular-weight iron present in the acidic vacuome may then be converted into a form which does not catalyze oxidative reactions, e.g., the formation of $HO^{\bullet}$ by homolytic cleavage of H_2O_2, at least not until the autophagocytosed ferritin is completely degraded. Partly degraded ferritin forms haemosiderin which has also been considered a substance capable of keeping ferric iron in a non-redox-active form.[33]

In this study we found that lysosomes were destabilized against oxidative stress as a result of iron uptake, with resultant decreased cellular survival when exposed to H_2O_2, but only in a situation when their cytosolic amounts of ferritin were comparatively low. On the other hand, in a situation where the cells initially contained increased amounts of cytosolic ferritin, the same lysosomal uptake of iron did not result in such a destabilization. To investigate the relationship between lysosomal iron, induction of ferritin synthesis, and cellular resistance to oxidative stress, we have employed a cell culture model

where lysosomal iron-loading was induced by non-physiological means. However, the findings are of potential importance also for the understanding of a number of pathophysiological conditions where increased amounts of lysosomal iron occur; such as haemochromatosis with resultant degeneration of hepatocytes and B-cells leading to cirrhosis and diabetes. It is well known that many years may pass before the parenchymal cells become critically damaged during haemochromatosis. The explanation may be related to mechanisms of the type described in this study, i.e., a chronic increase in lysosomal iron may be compensated for by autophagocytosis of apoferritin (or iron-unsaturated ferritin). Perhaps it is only when the lysosomal iron-loading is so substantial that ferritin autophagocytosis no longer binds all lysosomal iron in a non-redox-active form, that intralysosomal iron-catalyzed oxidative reactions reach a critical level and cause damage that is not repairable.

In conclusion, we suggest that in conditions of iron overload, induction of ferritin synthesis, with its concomitant autophagocytotic transport to the acidic vacuolar apparatus, enhances lysosomal stability by limiting oxidative membrane damage. This represents a novel cellular strategy for protection against oxidative stress.

Acknowledgment

This work was supported by the Swedish Medical Research Council (Grant 4481).

References

[1] Britton, R.S., Ramm, G.A., Olynyk, J., Singh, R., O'Neill, R. and Bacon, B.R. (1994). Pathophysiology of iron toxicity. *Advances in Experimental Medicine and Biology*, **356**, 239–253.

[2] Yuan, X.M., Li, W., Olsson, A.G. and Brunk, U.T. (1996). Iron in human atheroma and LDL oxidation by macrophages following erythrophagocytosis. *Atherosclerosis*, **124**, 61–73.

[3] Halliwell, B. and Gutteridge, J.M.C. (1989). *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford, pp. 31–47.

[4] Eaton, J.W. (1996). Iron: the essential poison. *Redox Report*, **2**, 215.

[5] Garner, B., Li, W., Roberg, K. and Brunk, U.T. (1997). On the cytoprotective role of ferritin in macrophages and its ability to enhance lysosomal stability. *Free Radical Research*, **27**, 487–500.

[6] Brunk, U.T., Zhang, H., Roberg, K. and Öllinger, K. (1995). Lethal hydrogen peroxide toxicity involves lysosomal iron-catalyzed reactions with membrane damage. *Redox Report*, **1**, 267–277.

[7] Yuan, X.M., Brunk, U.T. and Olsson, A.G. (1995). Effects of iron- and hemoglobin-loaded monocyte-derived macrophages on oxidation and uptake of LDL. *Arteriosclerosis Thrombosis and Vascular Biology*, **15**, 1345–1351.

[8] Yuan, X.M., Olsson, A.G. and Brunk, U.T. (1996). Macrophage erythrophagocytosis and iron exocytosis. *Redox Report*, **2**, 9–17.

[9] Öllinger, K. and Roberg, K. (1997). Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. *Journal of Biological Chemistry*, **272**, 23 707–23 711.

[10] Loegering, D.J., Raley, M.J., Reho, T.A. and Eaton, J.W. (1996). Macrophage dysfunction following the phagocytosis of IgG-coated erythrocytes: production of lipid peroxidation products. *Journal of Leukocyte Biology*, **59**, 357–362.

[11] Harrison, P.M. and Arosio, P. (1996). The ferritins: molecular properties, iron storage function and cellular recognition. *Biochimica et Biopysica Acta*, **1275**, 161–203.

[12] Öllinger, K. and Brunk, U.T. (1995). Cellular injury induced by oxidative stress is mediated through lysosomal damage. *Free Radical Biology and Medicine*, **19**, 565–574.

[13] Hellquist, H.B., Svensson, I. and Brunk, U.T. (1997). Oxidant-induced apoptosis: a consequence of lethal lysosomal leak? *Free Radical Biology and Medicine*, **3**, 65–70.

[14] Selden, C., Owen, M., Hopkins, J.M.P. and Peters, T.J. (1980). Studies on the concentration and intracellular localization of iron proteins in liver biopsy specimens from patients with iron overload with special reference to their role in lysosomal disruption. *British Journal of Haematology*, **44**, 593–603.

[15] Zdolsek, J., Roberg, K. and Brunk, U.T. (1993). Visualisation of iron in cultured macrophages: a cytochemical light and electron microscopic study using autometallography. *Free Radical Biology and Medicine*, **15**, 1–11.

[16] Danscher, G. (1981). Localization of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry*, **71**, 81–88.

[17] Danscher, G. and Møller-Madsen, B. (1985). Silver amplification of mercury sulfide and selenide: A histochemical method for light and electron microscopic localization of mercury in tissue. *Journal of Histochemistry and Cytochemistry*, **33**, 219–228.

[18] Abok, K., Fredriksson, B.-A. and Brunk, U.T. (1988). An experimental model system for leishmaniasis. An ultrastructural study on cultured macrophages exposed to Leishmania parasites and sodium stibogluconate. *Acta Pathologica Microbiologica Immunologica Scandanavia*, **96**, 589–595.

[19] Panus, P.C., Radi, R., Chumley, P.H., Lillard, R.H. and Freeman, B.A. (1993). Detection of H_2O_2 release from vascular endothelial cells. *Free Radical Biology and Medicine*, **14**, 217–223.

[20] Abok, K., Hirth, T., Ericsson, J.L.E. and Brunk, U.T. (1983). Macrophage radiosensitivity in culture as a function of

exposure to ionic iron. *Virchows (Cellular Pathology)*, **42**, 119–129.
[21] Jonas, S.K. and Riley, P.A. (1992). Modification of the *in vitro* cytotoxicity of hydrogen peroxide by iron complexes. *Free Radical Research Communications*, **17**, 407–419.
[22] Abok, K., Hirth, T., Ericsson, J.L.E. and Brunk, U.T. (1983). Effect of iron on the stability of macrophage lysosomes. *Virchows (Cellular Pathology)*, **43**, 85–101.
[23] Moss, D., Hibbs, A.R., Stenzel, D., Powell, L.W. and Halliday, J.W. (1994). The endocytic pathway for H-ferritin established in live MOLT-4 cells by laser scanning confocal microscopy. *British Journal of Haematology*, **88**, 746–753.
[24] Braumann, A., Wulfhekel, U., Nielson, P., Balkenhol, B. and Dullmann, J. (1994). Pattern of iron storage in the rat heart following iron overloading with trimethylheaxanoyl-ferrocene. *Acta Anat. Basel*, **150**, 45–55.
[25] Olakanmi, O., McGowan, S.E., Hayek, M.B. and Britigan, B.E. (1993). Iron sequestration by macrophages decreases the potential for extracellular hydroxyl radical formation. *Journal of Clinical Investigation*, **91**, 889–899.
[26] Aisen, P. (1977). Some physicochemical aspects of iron metabolism. In: *Iron Metabolism*. Ciba Foundation Symposium 51, Amsterdam, Elsevier, pp. 1–18.
[27] Pisoni, R.L., Acker, T.L., Lisowski, K.M., Lemons, R.M. and Thoene, J.G. (1990). A cysteine-specific lysosomal transport system provides a major route for the delivery of thiol to human fibroblast lysosomes: possible role in supporting lysosomal proteolysis. *Journal of Cell Biology*, **110**, 327–335.
[28] Breuer, W., Greenberg, E. and Cabantchick, Z.I. (1997). Newly delivered transferrin iron and oxidative cell injury. *Federation of European Biochemical Societies Letters*, **403**, 213–219.
[29] DeDuve, C. and Wattiaux, R. (1966). Functions of lysosomes. *Annual Review of Physiology*, **28**, 435–492.
[30] Lawson, D.M., Treffry, A., Artymiuk, P.J., Harrison, P.M., Yewdall, S.J., Luzzago, A., Cesarini, G., Levi, S. and Arosio, P. (1989). Identification of the ferroxidase centre in ferritin. *Federation of European Biochemical Societies Letters*, **287**, 207–210.
[31] Bolann, B.J. and Ulvik, R.J. (1990). On the limited ability of superoxide to release iron from ferritin. *European Journal of Biochemistry*, **193**, 899–904.
[32] Mortimore, G.E., Miotto, G., Venerando, R. and Kadowaki, M. (1996). Autophagy. In: *Subcellular Biochemistry, Vol. 27: Biology of the Lysosome*. Lloyd, T.B. and Mason, R.W., eds., New York, Plenum Press, pp. 93–135.
[33] O'connell, M., Halliwell, B., Moorhouse, C.P., Aruoma, O.I., Baum, H. and Peters, T.J. (1986). Formation of hydroxyl radicals in the presence of ferritin and haemosiderin. Is haemosiderin formation a biological protective mechanism? *Biochemical Journal*, **234**, 727–731.