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Subcellular localization of ferritin and iron taken up by rat hepatocytes

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The subcellular localization of ferritin and its iron taken up by rat hepatocytes was investigated by sucrose-density-gradient ultracentrifugation of cell homogenates. After incubation of hepatocytes with ^{125}I-labelled [^{59}Fe]ferritin, cells incorporate most of the labels into structures equilibrating at densities where acid phosphatase and cytochrome *c* oxidase are found, suggesting association of ferritin and its iron with lysosomes or mitochondria. Specific solubilization of lysosomes by digitonin treatment indicates that, after 8 h incubation, most of the ^{125}I is recovered in lysosomes, whereas ^{59}Fe is found in mitochondria as well as in lysosomes. As evidenced by gel chromatography of supernatant fractions, ^{59}Fe accumulates with time in cytosolic ferritin. To account for these results a model is proposed in which ferritin, after being endocytosed by hepatocytes, is degraded in lysosomes, and its iron is released and re-incorporated into cytosolic ferritin and, to a lesser extent, into mitochondria.

INTRODUCTION

After erythrophagocytosis, Kupffer cells [1], peritoneal macrophages [2] and bone-marrow macrophages [3] release much of the iron that they have ingested to the extracellular medium in the form of ferritin. Hepatocytes, which display specific ferritin receptors on their surface [4,5], are capable of taking up this ferritin from the medium bathing the cells [6]. Although previous results [6] suggest that hepatic lysosomes might play an important role in the intracellular release of ferritin iron by freeing the metal after degradation of the protein, the metabolic pathways taken by ferritin and its iron in the cell remain largely unknown. We have taken advantage of cell fractionation techniques [7] and the specific solubilization of lysosomes by digitonin treatment [8] to investigate further the fate of ferritin and its iron after uptake by rat hepatocytes.

MATERIALS AND METHODS

Proteins and cells

Rat liver ferritin was prepared by the method of Penders *et al.* [9] after being labelled *in vivo* with ^{59}Fe by the procedure of Niitsu *et al.* [10]. [^{125}I]Iodinated ferritin was prepared with the Bolton–Hunter reagent (ICN Radiochemicals, Irvine, CA, U.S.A.) as described in ref. [11]. Hepatocytes were isolated, plated and incubated as reported in ref. [6].

Cell fractionation

After incubation at 37 °C, 1.2×10^7 cells were washed five times at 4 °C with chilled 0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, scraped off and homogenized in a final volume of about 10 ml of chilled 0.25 M-sucrose/3 mM-imidazole/HCl buffer, pH 7, in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.). The nuclear fraction (N) was separated from the cytoplasmic extract by centrifugation at 1000 *g* for 10 min at 4 °C (IEC centrifuge, rotor 269) and washed twice. The cytoplasmic extract was further separated into MLP and S fractions by centrifugation at 100000 *g* for 30 min at 4 °C in a Ti 65 rotor (Beckman) as described in ref. [7]. The MLP fraction was then resuspended in 0.25 M-sucrose/3 mM-imidazole/HCl buffer, pH 7, which was supplemented with sucrose (1.34 g/ml) to reach a density of 1.27 g/ml. This suspension was applied at the bottom of a centrifuge tube with a linear sucrose gradient of density 1.06–1.24 g/ml, and centrifuged at 200000 *g* for 90 min at 4 °C in a VTI 50 rotor (Beckman). After centrifugation 14 fractions were collected, weighed and analysed for radioactivity and enzyme activity. Activity of 5′-nucleotidase was assayed as described in ref. [12], acid phosphatase as described in ref. [13] and cytochrome *c* oxidase as described in ref. [14].

Digitonin treatment of the ML fraction

The ML fraction was obtained by centrifuging the cytoplasmic extract at 40000 *g* for 5 min at 4 °C in a Ti 65 rotor (Beckman). The fraction was incubated with 0.3 mg of digitonin (Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml for 10 min at 0 °C as described in ref. [8].

RESULTS

Distribution of marker enzymes, ferritin and iron in the N, MLP and S fractions

Hepatocytes were incubated for 1 h, 3 h and 8 h at

Abbreviations used: N, nuclear fraction; M, mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, final supernatant; ML, combined M and L fractions; MLP, combined M, L and P fractions.

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Table 1. Distribution of marker enzymes and ^{125}I-labelled [^{59}Fe]ferritin among the N, MLP and S fractions

Hepatocytes were incubated for 1 h, 3 h and 8 h in culture medium containing 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml. After homogenization and fractionation of the cells, the N, MLP and S fractions were analysed for radioactivity and for enzyme content. Values represent mean percentages of total enzyme activity or radioactivity recovered from three independent experiments ±S.D. Abbreviation: N.D., not detected.

	Amount in fraction (% of total recovered)		
Enzyme or label	N fraction	MLP fraction	S fraction
Acid phosphatase	10.0±0.8	63.4±0.6	26.5±0.4
Cytochrome c oxidase	8.8±1.3	90.7±1.3	N.D.
5′-Nucleotidase	38.4±2.3	57.4±2.7	4.25±0.4
^{59}Fe (1 h)	10.7±0.6	65.0±8.7	24.3±1
^{59}Fe (3 h)	11.8±0.8	56.7±4.6	31.3±1.8
^{59}Fe (8 h)	10.4±0.6	55.7±6	34.0±3.2
^{125}I (1 h)	13.5±1.4	67.6±12.4	18.9±1.2
^{125}I (3 h)	14.0±1.1	73.8±2.9	12.1±0.2
^{125}I (8 h)	13.8±0.7	75.8±5.3	10.4±2.2

37 °C in the presence of 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml, then washed, homogenized and fractionated to obtain N, MLP and S components. After 8 h incubation, 6% of the ^{59}Fe and 2% of the ^{125}I originally present in the culture medium were recovered in the cells. As shown in Table 1, activities of acid phosphatase, cytochrome *c* oxidase and 5′-nucleotidase, as markers of lysosomes, mitochondria and plasma membrane respectively, are mostly recovered in the MLP fraction. Some acid phosphatase is also detected in the S fraction, and 5′-nucleotidase is present as well in the N fraction. At all incubation times, the bulk of ^{59}Fe and ^{125}I is found in the MLP fraction. The relative amount of ^{59}Fe in the S fraction increases with time (from 24% of the total ^{59}Fe in the MLP fraction after 1 h incubation to about 34% after 8 h of incubation of the MLP fraction), indicating an iron enrichment of the S fraction as the experiment progresses. Only small amounts of ^{125}I label are found in N and S fractions.

Density distribution of marker enzymes, ^{59}Fe and ^{125}I in the MLP fraction

The density distributions of the marker enzymes acid phosphatase, cytochrome *c* oxidase and 5′-nucleotidase and the radioactive labels ^{59}Fe and ^{125}I were analysed by isopycnic centrifugation. As shown in Fig. 1, acid

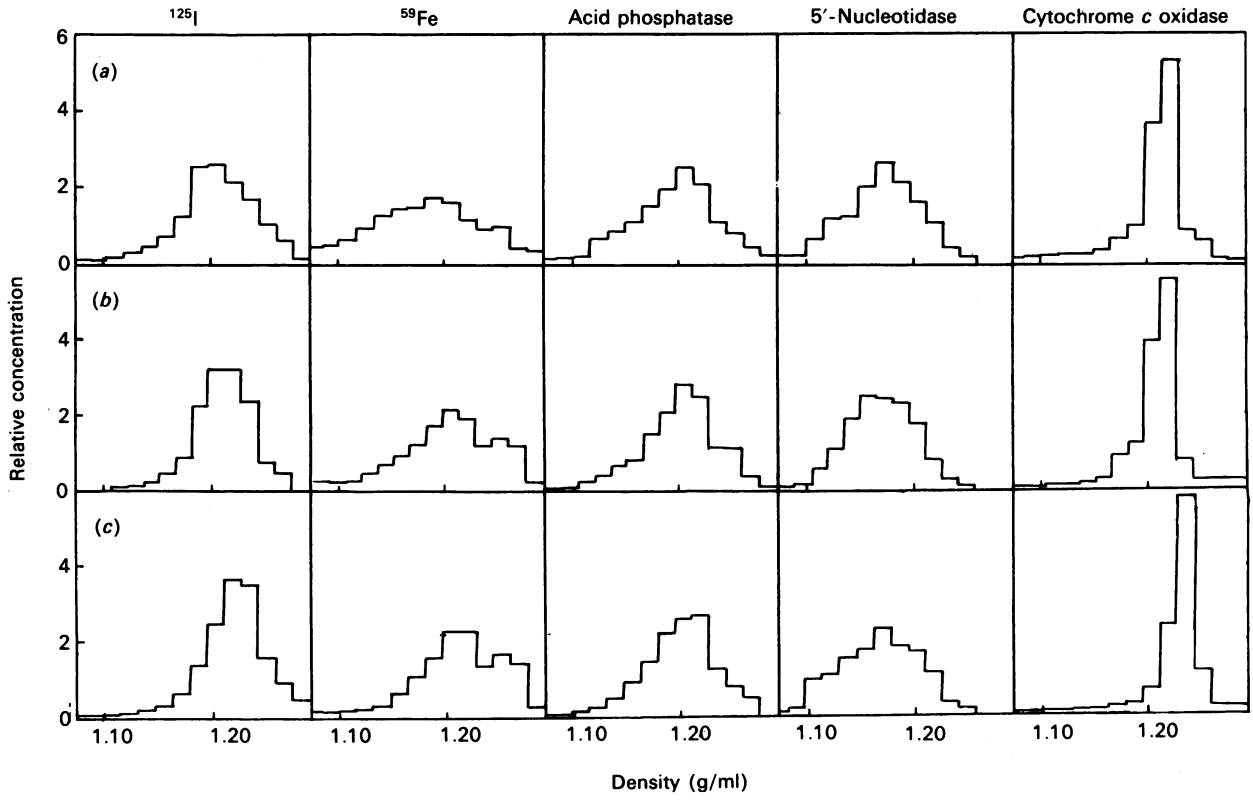

Fig. 1. Density distribution of ^{125}I-labelled [^{59}Fe]ferritin, acid phosphatase, 5′-nucleotidase and cytochrome *c* oxidase in the N, MLP and S fractions

Hepatocytes were incubated at 37 °C in culture medium containing 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml for 1 h (*a*), 3 h (*b*) and 8 h (*c*), then taken for fractionation.

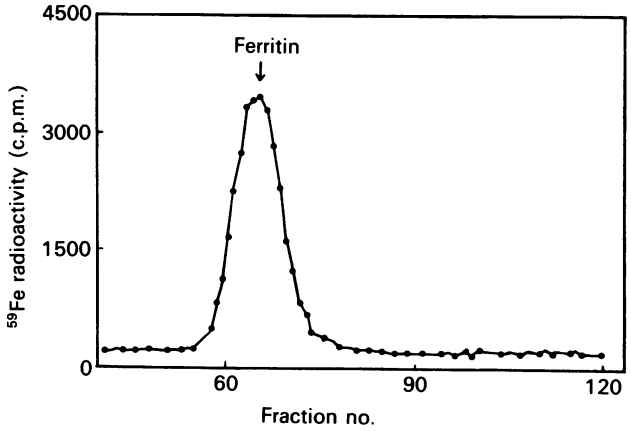

Fig. 2. Chromatogram of the S fraction

Cells were incubated with medium containing 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml for 8 h at 37 °C, homogenized and fractionated. The S fraction was then chromatographed on a column of Ultrogel AcA 34. Arrow indicates mobility of a rat liver ferritin standard.

phosphatase mainly equilibrates at sucrose densities of 1.21 g/ml, cytochrome *c* oxidase is mostly recovered at a density of 1.23 g/ml and 5'-nucleotidase is found at a density of 1.17 g/ml. At all incubation times, ^{125}I is mainly detected at densities around 1.21–1.22 g/ml, where acid phosphatase and cytochrome *c* oxidase are found. After 1 h incubation ^{59}Fe radioactivity is mostly found in the middle of the gradient, where 5'-nucleotidase activity is present, but where acid phosphatase also localizes. However, at this time, some ^{59}Fe and ^{125}I are also present at lower sucrose densities. Longer incubation periods show association of ^{59}Fe with structures sedimenting at densities of 1.22 g/ml and bearing acid phosphatase and/or cytochrome *c* oxidase activity.

Analysis of the S fraction obtained after differential centrifugation

As indicated in Table 1, about 35% of the ^{59}Fe present in cells incubated for 8 h at 37 °C is recovered in the S fraction. Analysis of the S fraction by gel chromatography shows a single ^{59}Fe peak corresponding in mobility to a standard of rat liver ferritin (Fig. 2).

Digitonin treatment of the ML fraction

The density distribution experiments of ^{125}I-labelled [^{59}Fe]ferritin presented in Fig. 1 indicate that ^{59}Fe and ^{125}I are essentially detected at sucrose densities where acid phosphatase and cytochrome *c* oxidase equilibrate, suggesting their association with lysosomes and/or mitochondria. To quantify the amounts of ^{59}Fe and ^{125}I present in the lysosomes an ML fraction, obtained from cells incubated for 8 h with 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml, was treated with 0.3 mg of digitonin/ml. This released 52% of the total acid phosphatase activity, 31% of the ^{59}Fe and 46% of the ^{125}I. Digitonin treatment does not significantly affect mitochondrial integrity, since only 3% of the total cytochrome *c* oxidase present in the ML fraction was solubilized.

DISCUSSION

Hepatocytes incubated with 4 μg of ^{125}I-labelled [^{59}Fe]ferritin/ml incorporate most of the label in cell structures comprising the MLP fraction. After isopycnic sedimentation of the MLP fraction ^{59}Fe and ^{125}I equilibrate in the sucrose gradient with acid phosphatase and cytochrome *c* oxidase, suggesting association of the isotopes with lysosomes, mitochondria, or both. At 1 h ^{125}I and ^{59}Fe are also found at low sucrose densities, consistent with their presence in endosomes. Digitonin treatment of the ML fraction releases acid phosphatase, ^{59}Fe and ^{125}I, indicating the presence of the radioactive labels in lysosomes. Should all lysosomes have been solubilized, we estimate that about 60% of the ^{59}Fe and 90% of the ^{125}I present in the ML fraction would have been released after digitonin treatment. Whereas most of the ^{125}I label seems to be associated with lysosomes, about 40% of the ^{59}Fe still sediments with digitonin-resistant cell structures, presumably mostly mitochondria. However, exact quantification of the amount of iron present in mitochondria requires further purification of these organelles, since ^{59}Fe could also be associated with plasma-membrane fragments, which are still present, although in small amounts, in the ML fraction.

^{125}I radioactivity in the S fraction averages 14% of the total ^{125}I radioactivity in the MLP fraction, but ^{59}Fe content increases from 24% after 1 h incubation to 34% after 8 h incubation. As evidenced by gel chromatography, most of this ^{59}Fe in the S fraction is in cytosolic ferritin. Very probably this represents endogenous ferritin, since the ratio of ^{59}Fe to ^{125}I increases progressively with time.

Consistent with this study, and previous reports pointing to the importance of lysosomes in the degradation of extracellular ferritin [15–17], is a mechanism of ferritin uptake by hepatocytes that entails endocytosis of the protein, its transfer to lysosomes, where protein degradation and release of iron occur, and subsequent reincorporation of the metal into endogenous cytosolic ferritin. To a lesser extent, ferritin-borne iron is also available to mitochondria. Contrasting with this pathway is the transferrin cycle of hepatocytes, in which lysosomal digestion of the protein does not seem to be necessary for release of intracellular iron [18,19].

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