IRON MOBILIZATION FROM CULTURED RAT FIBROBLASTS AND HEPATOCYTES

Effect of various drugs

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1. Introduction

Up to now, the mechanisms involved in the cell biology of iron metabolism remain poorly understood. Cultured fibroblasts take up transferrin through receptor-mediated endocytosis, iron is released inside lysosomes and the iron-depleted protein is returned intact to the extracellular medium [1–3]. No clear evidence has been reported on the mechanisms whereby iron is released from cells in which it is mainly present in haem, bound to low molecular mass ligands or is stored in ferritin. However, several diseases are characterized by an iron overload which is predominantly localized in the hepatocytes. Ceruloplasmin could play an important role [4] in hepatic iron mobilisation, although recent in vitro studies suggest that ceruloplasmin does not influence transferrin formation nor iron transfer between ferritin and transferrin [5]. To better understand this physiopathological process, we have compared the mobilisation of iron from 2 different cell types in various conditions.

2. Materials and methods

2.1. *Purification and labelling of proteins*

Transferrin was isolated from rat serum as in [3]. Apotransferrin was prepared by dialysis against 0.3 M sodium acetate buffer (pH 5.5) containing 0.01 M EDTA and then against PBS (phosphate-buffered saline: 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4). Rat liver ferritin was isolated as in [6]. Antibodies against transferrin and ferritin were raised in rabbits as in [3]. Ceruloplasmin was isolated from rat serum by chromatography on DEAE-Sephadex and gel filtration on Sephadex G-150 as in [7]. Rat serum was dialyzed against 0.1 M sodium acetate buffer (pH 5.5) containing 0.05 M EDTA, then against distilled water and finally adjusted to pH 7.5 with sodium bicarbonate. It was then loaded with ^{59}Fe as ferric citrate in order to have a specific activity close to 10 000 dpm/μg transferrrin (assuming a serum concentration of 3 mg/ml) and finally saturated with a 5-fold excess of [^{56}Fe]ferric citrate. In these conditions more than 95% of the ^{59}Fe was retained by anti-transferrin antibody bound to Sepharose–protein A.

2.2. *Cell culture*

Primary cultures of rat embryo fibroblasts were subcultivated to confluence as in [8] in 20 cm^2 plastic Petri dishes. Rat hepatocytes were isolated as in [9]; cells were plated on collagen-coated gas-permeable hydrophobic Petri dishes. The toxicity of the drugs used was determined after incubation of cells with different drug concentrations and observation by phase-contrast microscopy. The concentration selected was the highest one at which no morphological alteration was observed in the course of a 72 h incubation at 37°C.

Cultured fibroblasts or hepatocytes were incubated for 16 h at 37°C in 1 ml of culture medium containing 10 and 15% of ^{59}Fe-loaded rat serum, respectively. The cells were then washed 5 times with 1 ml of PBS,

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once with 1 ml culture medium and twice with 1 ml PBS and then reincubated for different times in fresh culture medium. At the end of this reincubation the cells were washed similarly and finally resuspended in 2 ml of 1% Triton X-100 (w/v) in PBS. Aliquots were assayed for radioactivity and protein as in [3]. The amount of cell-associated ^{59}Fe which reacts with anti-ferritin antibody was determined as in [3].

3. Results and discussion

3.1. *Iron mobilization in the absence of drugs*

Cultured fibroblasts were incubated for 16 h with 10% rat serum, the transferrin of which had been charged with ^{59}Fe; the cells were then washed and reincubated for various times in fresh culture medium. When fibroblasts were reincubated in fresh culture medium containing 10% calf serum, ^{59}Fe was progressively released up to 48 h and thereafter a plateau corresponding to 40% of the initial level was reached (fig.1A). At the beginning of the reincubation 48% of the cell-associated ^{59}Fe reacted with anti-ferritin antibody immobilized on Sepharose–protein A; this value fell to 26% after 48 h and then remained constant. When the reincubation medium was supplemented with 100 μg/ml of rat apotransferrin or contained, instead of calf serum, 10% fresh rat serum (in which the ferroxidase activity of ceruloplasmin assayed according to [10] was still present) and 100 μg/ml apotransferrin, the amount of ^{59}Fe released from the fibroblasts was not significantly different from control values (not shown).

In another series of experiments, ^{59}Fe-loaded hepatocytes, in which almost all the label had been incorporated into cytosol ferritin, as determined by reaction with anti-ferritin antibody and cell fractionation techniques [11], were reincubated in fresh culture medium either as such (fig.1B) or with apotransferrin (130 μg/ml) in the presence or the absence of 540 μg/ml of rat ceruloplasmin. Whatever the experimental conditions, no significant amount of ^{59}Fe was released from the cells even after 72 h reincubation.

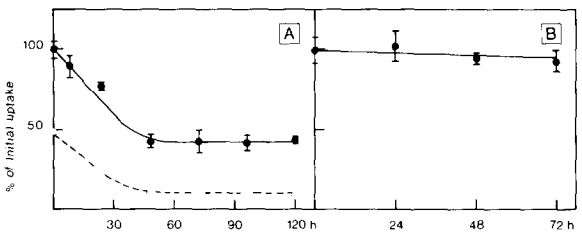

Fig.1. Rat fibroblasts (A) or hepatocytes (B) charged with ^{59}Fe were washed and reincubated for different times in fresh culture medium. At the end of the reincubation, the cells were washed and analysed (see section 2). •, ^{59}Fe associated with the cells (mean ± S.D. of 3 independent experiments); ○, ^{59}Fe reacting with anti-ferritin antibody bound to Sepharose–protein A.

These results indicate that part of the ^{59}Fe accumulated by fibroblasts, even when it is incorporated into ferritin, can rather easily be mobilized from fibroblasts. In contrast when iron is stored in the ferritin of cultured hepatocytes, it cannot be released from the cells. On the other hand, our data indicate that in contrast to what was reported [4], the presence of ceruloplasmin does not increase the mobilization of iron from cultured liver cells.

3.2. *Effect of drugs which affect endocytosis*

The effect of several drugs which either increase the intralysosomal pH [12] or affect endocytosis [13,14] on iron mobilization has been tested. Drugs such as methylamine, chloroquine and amantadine considerably diminish the uptake of iron from iron-saturated transferrin by cultured fibroblasts and hepatocytes [1,2].

However, as indicated by fig.2, none of these drugs nor monodansylcadaverine, a strong inhibitor of the transglutaminase activity, which seems to be involved in receptor-mediated endocytosis [15], significantly affect the mobilization of iron from cultured cells. These data suggest that neither endocytosis nor lyso-

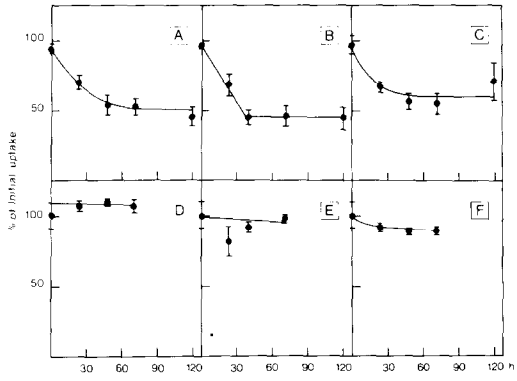

Fig.2. Rat fibroblasts (A–C) or hepatocytes (D–F) charged with ^{59}Fe were washed and reincubated for different times in a fresh culture medium containing 10 mM (A) or 1 mM (D) methylamine; 100 μM (B) or 50 μM (E) chloroquine, 200 μM monodansylcadaverine (C) or 50 μM colchicine (F). At the end of the reincubation, cells were washed and analysed (see section 2). Mean ± S.D. of 3 independent experiments.

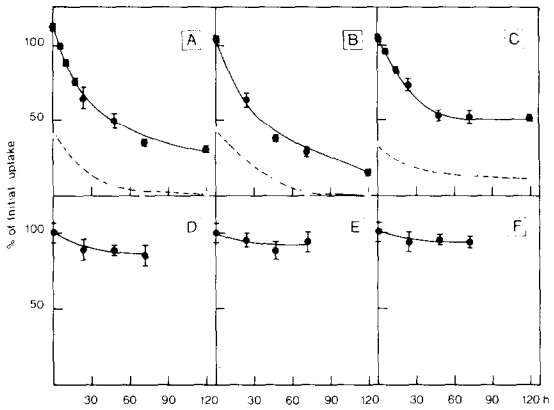

Fig.3. Rat fibroblasts (A–C) or hepatocytes (D–F) charged with ^{59}Fe were washed and reincubated for different times in a fresh culture medium containing 50 μM desferrioxamine B (A,D), 500 μM rhodotorulic acid (B,E), 1 mM 2,3-dihydroxybenzoate (C) or 50 μM bipyridyl (F). At the end of the incubation, cells were washed and analyzed (see section 2). •, ^{59}Fe associated with the cells (mean ± S.D. of 3 independent experiments); ○, ^{59}Fe reacting with anti-ferritin antibody bound to Sepharose–protein A.

somal function which are both affected by these drugs are involved in the process of iron mobilization.

3.3. *Effect of iron chelators*

Iron chelators such as desferrioxamine B, rhodotorulic acid, or bipyridyl greatly reduce the uptake of ^{59}Fe from iron-saturated transferrin by cultured fibroblasts or hepatocytes [1,2]. When the reincubation medium of fibroblasts preincubated for 16 h at 37°C with ^{59}Fe-loaded rat serum was supplemented with iron chelators such as 50 μM desferrioxamine B, 500 μM rhodotorulic acid or 50 μM 2,2′-bipyridyl (not shown), more iron was released from the cells than from controls (fig.3). Neither 1 mM 2,3-dihydroxybenzoate nor 100 μM isoniazidpyridoxal hydrazone (not shown) had any effect. The kinetics of mobilization were comparable to control experiments up to 48 h reincubation, but thereafter iron release continued up to 120 h; at this time, 15% of the ^{59}Fe still remained bound to the cells and did not react with anti-ferritin antibody. In contrast, when cultured hepatocytes precharged with ^{59}Fe are reincubated in a fresh medium supplemented with these iron chelators, none of them appears able to mobilize ^{59}Fe from the cells.

4. Conclusions

In cultured fibroblasts an important proportion of ^{59}Fe taken up from transferrin, even when it is incorporated in ferritin can be progressively released in a fresh culture medium containing serum. Iron mobilization is not affected by the addition of ceruloplasmin nor by apotransferrin; it is, however, increased by several iron chelators such as desferrioxamine B, rhodotorulic acid or 2,2′-bipyridyl, but not by others. In contrast in cultured hepatocytes where almost all the cell-associated ^{59}Fe is incorporated into ferritin, whatever the experimental conditions, iron cannot be released.

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