

Ceruloplasmin, transferrin and apotransferrin facilitate iron release from human liver cells

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Received 7 April 1997

Abstract The rate of iron release from HepG2 liver cells was increased not only by extracellular apotransferrin, but also by diferric transferrin, in a non-additive, concentration-dependent manner and to a similar magnitude. This suggests that rapid equilibration between receptor-mediated uptake and the release process determines net iron retention by the liver. Release was also accelerated by ceruloplasmin; most importantly, the effect of this protein was greatest when iron release was occurring rapidly, stimulated by apotransferrin, or under conditions of limited oxygen. Thus iron release involves both apotransferrin and ferrotransferrin, with ceruloplasmin playing a role in tissues with limited oxygen supply, as in the liver in vivo.

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Key words: Iron metabolism; Transferrin; Ceruloplasmin; Ferroxidase; HepG2 cell

1. Introduction

Ceruloplasmin, the copper-containing serum protein, has been postulated as the critical ferroxidase for over 30 years. Whilst there is no doubt that oxidation is necessary, and that ceruloplasmin is biochemically capable of this role, there has been little evidence to date that this occurs in a cellular or in vivo environment.

Recent reports of hepatic accumulation of iron stores in patients with a hereditary ceruloplasmin deficiency [1] have confirmed that this copper-containing ferroxidase plays a role in mobilizing iron from tissues stores. A number of early animal studies also demonstrated that this was likely to be the case, for instance Osaki et al. [2] showed that it could accelerate iron release from the perfused liver, while the iron-binding protein apotransferrin alone appeared to have little effect. However, these experiments did not address the question of whether the ceruloplasmin-catalyzed release required apotransferrin as an acceptor. Free iron is inherently toxic, being a potent catalyst for the synthesis of free oxygen species; in vivo, therefore, iron is usually complexed to either transferrin or ferritin. In previous work investigating the mechanisms of iron homeostasis in isolated rat hepatocytes, we found that release from hepatocytes of newly acquired iron could occur simultaneously with the uptake process and that this release could be accelerated by the addition of apotransferrin [3], and yet we found no evidence for the presence of receptors specific

for apotransferrin on these cells. In support of this role for transferrin in iron release, Baker et al. showed that the release of iron from freshly isolated rat hepatocytes, labelled in vivo with ⁵⁹Fe, was accelerated by chelators, serum and apotransferrin [4], although the transferrin effect was not observed by all workers [5].

The oxidation state of the iron is a crucial factor in its mobilization. Iron is bound to transferrin in the ferric, iron (III) state, yet most intracellular iron is released from ferritin, the primary intracellular iron storage protein, in the ferrous iron (II) form. An oxidation step is therefore required before this iron may be effectively mobilized outside the cell.

We were therefore lead to re-examine the release of iron from liver cells, and to determine how both transferrin and ceruloplasmin influence the release. Here, we show that both iron- and apo- transferrin accelerate iron release from the human hepatoma cell line HepG2, and that this release rate is greatly enhanced by ceruloplasmin. Interestingly, this enhancement is greatest under conditions of low oxygen tension, and therefore where the rate of spontaneous iron oxidation is lowest.

2. Materials and methods

2.1. Materials

Media, sera and tissue culture plastics were obtained from Life Technologies (Paisley, Scotland, UK) while bovine serum albumin (BSA, fraction V), was from Sigma Chemical Co. (Poole, Dorset, UK). The bovine albumin was shown to be free of transferrin by radial immunodiffusion against anti-bovine transferrin antisera (rabbit) and by the absence of iron-binding capacity using the ferrozine method [6]. Chelex 100 was bought from BioRad Laboratories (Bromley, Kent, UK) and human ceruloplasmin from Green Cross Corp. (Osaka, Japan). This source of ceruloplasmin was the highest quality available; the protein was verified by us to migrate as a single band on electrophoresis and to have an A_{640}/A_{280} of 0.047, comparable to highly purified protein previously described [7]. Unless otherwise stated other chemicals were of analytical grade from Fisher Scientific (Loughborough, UK).

2.2. Cells

The human hepatoma cell line, HepG2 [8], was grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), penicillin, streptomycin and glutamate. Cells were removed from dishes using trypsin/EDTA, washed 3 times in MEM with 10% FBS, counted and 3×10^6 cells were added to 6-well polystyrene plates (10 cm²) 24 h before use. After experimental incubations cell viability was checked using trypan blue exclusion and lactate dehydrogenase release (Sigma LDH colorimetric assay) and was never less than 95%.

2.3. Transferrin labelling

Human apotransferrin (Boehringer-Mannheim, Lewes, UK) was loaded to 100% saturation with ⁵⁹FeCl₃ (Amersham International plc, Amersham Bucks) using a 20-fold excess of citrate to complex the iron as described by Cavill [9] and an ion exchange resin to remove uncomplexed iron citrate. Specific activity of the ⁵⁹Fe-transferrin was 1.1–1.2 cpm/pg Fe (1500 cpm/g transferrin protein). The

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methods used to label human diferric transferrin with ^{125}I (Amersham International, Bucks, UK) have been described in full elsewhere [10].

2.4. Transferrin binding, iron uptake and cell loading

Analysis of ^{125}I -labelled transferrin binding to adherent cells was done as described before [11]. To characterize iron uptake ^{59}Fe -transferrin (40 $\mu\text{g}/\text{ml}$) was added to triplicate wells (3×10^6 cells/well) in MEM/1% BSA (1 ml) under 5% CO_2/air and uptake followed for up to 6 h. Uptake was stopped by the addition of ice-cold phosphate-buffered saline, the wells were washed 3 times and cells dissolved in NaOH for counting in the γ -counter. To pre-load HepG2 with radio-iron, ^{59}Fe -transferrin was added to cells in 6-well plates to give a final concentration of 25 $\mu\text{g}/\text{ml}$ diferric transferrin. Incubation was continued for 18 h at 37°C in an atmosphere of 5% CO_2/air , after which the cells were cooled to 4°C and washed 3 times in cold medium containing 1% BSA. Using this procedure the total ^{59}Fe loaded into the samples of 3×10^6 cells in the experiments shown was in the range 9402–25 384 cpm (8.2–22.1 ng Fe).

2.5. Cell incubation for iron release

MEM containing 1% BSA (2 ml), which had previously been passed through a column of Chelex-100 to remove any iron, as described previously [12] was added to dishes containing cells pre-loaded with ^{59}Fe , following two washes in medium. The cells were incubated for two periods of 15 min in fresh medium to remove any residual labelled transferrin. Fresh medium (1 ml) was added together with test agents, to triplicate wells, and the cells were placed in an 5% CO_2/air atmosphere at 37°C for incubation for up to 24 h. For experiments at low oxygen tension, cells were cultured in sealed flasks containing 10% air/N_2 . The experiments were repeated on at least three separate occasions. The ^{59}Fe content of the supernatant was analyzed by counting samples of the supernatant (0.5 ml), in an LKB/Wallac RackGamma counter. Total ^{59}Fe content of a sample of the cells was also measured.

3. Results

The HepG2 cells used for these experiments, which expressed 1.4×10^5 transferrin receptors per cell, were loaded with ^{59}Fe using labelled diferric transferrin, uptake from which occurred specifically via the receptors, since 96% was blocked by a 20-fold excess of unlabelled diferric transferrin (data not shown). Subsequent release of this labelled iron was linear with time when cells were incubated in medium alone or in medium containing apotransferrin. Fig. 1 shows the release of iron over a 24 h period, and it is apparent that iron release occurs even in the absence of transferrin or serum, but that

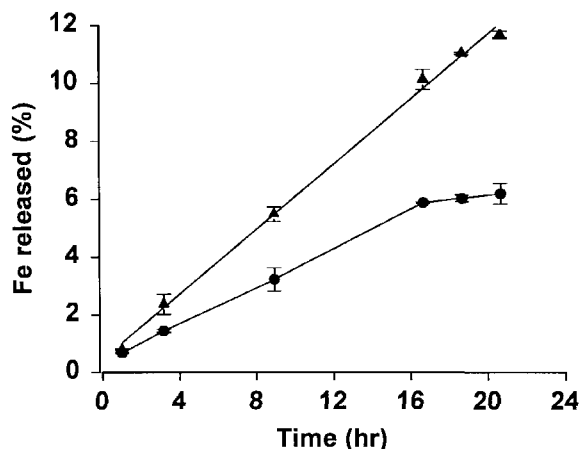


Fig. 1. Time course of iron release from HepG2 cells. Cells were pre-loaded with ^{59}Fe for 18 h, washed and then re-incubated in fresh iron-free MEM containing 1% BSA with (▲) or without (●) human apotransferrin (200 $\mu\text{g}/\text{ml}$). Shown is the mean $\pm$ SD ($n=3$) of the release at each time point.

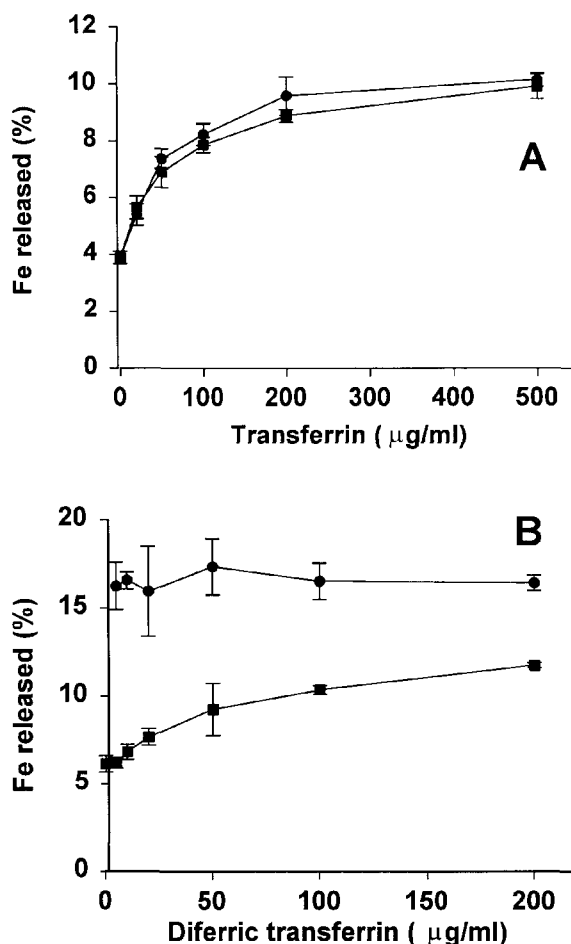


Fig. 2. Effect of apotransferrin and diferric transferrin on iron release from HepG2 cells. (A) After pre-loading, cells were incubated for 18 h with increasing concentrations of apotransferrin (●) or diferric transferrin (■). (B) As with (A) but cells were incubated with increasing diferric transferrin with (●) or without apotransferrin (■) at 500 $\mu\text{g}/\text{ml}$ present throughout.

the addition of apotransferrin accelerates the release by approximately 40%.

The effect of apotransferrin was further investigated by culturing cells overnight in medium containing increasing concentrations of apotransferrin. The effect of apotransferrin was concentration-dependent and saturable, with the maximum effect being reached with 200 $\mu\text{g}/\text{ml}$ apotransferrin (Fig. 2A). Unexpectedly, however, when this experiment was performed using iron-saturated transferrin, a very similar effect on iron release was observed (Fig. 2A).

Experiments were therefore performed to determine if the iron-releasing effects of apo- and diferric transferrin were additive. Cells in the presence or absence of an excess of apotransferrin were incubated in increasing concentrations of diferric transferrin. Again the effect of diferric transferrin alone approached saturation by 200 $\mu\text{g}/\text{ml}$ (Fig. 2B); however, there was no additional effect of diferric transferrin when excess apotransferrin (500 $\mu\text{g}/\text{ml}$) was present throughout. When the experiment was reversed and apotransferrin was titrated in the presence of excess diferric transferrin, a very similar picture was observed (data not shown), suggesting that there is a single pool of iron from which either apo- and diferric transferrin can accelerate release.

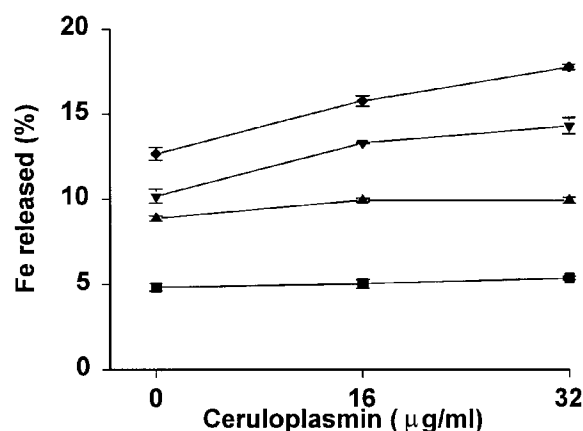


Fig. 3. Effect of apotransferrin and ceruloplasmin on iron release from HepG2 cells. Pre-labelled cells were incubated with two concentrations of ceruloplasmin and apotransferrin at 0 (■), 100 (▲), 200 (▼) or 500 (◆) µg/ml for 18 h and the release of ^{59}Fe monitored.

The effects of ceruloplasmin in this *in vitro* system were then investigated by adding human ceruloplasmin to the cell cultures. Fig. 3 shows that when iron was released into medium containing little (100 µg/ml) or no apotransferrin, ceruloplasmin did not significantly affect the release rate. However, when the background rate of release was accelerated, by the addition of larger amounts of apotransferrin, then there was a significant effect elicited by ceruloplasmin. At 500 µg/ml transferrin, 32 µg/ml of ceruloplasmin increased iron release by 40% in the experiment shown. These results therefore indicate that ceruloplasmin was particularly active in conditions where the iron release from the cells was occurring rapidly.

It has been shown previously that release of iron from hepatic cells can be accelerated in conditions of low oxygen tension [4]; our preliminary experiments confirmed this also to be the case with the HepG2 cells (data not shown). We therefore investigated whether ceruloplasmin might also be more significant under conditions of limited oxygen. Incubation of cells under low oxygen with both apotransferrin (20 µg/ml) and ceruloplasmin showed an augmentation of iron release by nearly 40% of that caused by apotransferrin alone (Table 1). This is in contrast with the release at normal oxygen concentration, where ceruloplasmin had no discernable effect on release to medium containing 20 µg/ml apotransferrin. At low apotransferrin concentrations, therefore, the action of ceruloplasmin on iron release is significant only when limited oxygen is present.

Table 1
Effect of oxygen, ceruloplasmin and apotransferrin on iron release from HepG2 cells

Additions	% Control release
None	100 ± 15.5
Apotransferrin (20 µg/ml)	104.5 ± 3.4
Ceruloplasmin (32 µg/ml)	99.9 ± 5.4
Both	142.7 ± 10.4

^{59}Fe -loaded cells were incubated under 10% air/90% N_2 with the addition of apotransferrin and/or ceruloplasmin. Cell viability did not differ during the 3 h incubation. The level of release with both proteins added was significantly ($P < 0.05$) higher than the other values.

4. Discussion

We found that iron release from the HepG2 cells occurred at a constant rate over the course of 24 h (Fig. 1). This linear release has also been shown with both short-term [13] and long-term cultures of rat hepatocytes [5] and suggests rapid equilibration of the radiolabelled iron taken up from transferrin with the intracellular pools of iron.

The release of iron could be accelerated by the addition of apotransferrin (Figs. 1 and 2), supporting previous findings with both the perfused rat liver [14] and with suspensions of isolated hepatocytes [15]. However, in neither of these systems could release of iron be increased by increasing the apotransferrin concentration, suggesting that the rate of release is predominantly modulated by intracellular factors. Here, we found that the iron release could be greatly enhanced by adding more apotransferrin (Fig. 2A) and, perhaps more surprisingly, that diferric transferrin could also enhance the release (Fig. 2A). Others have shown such an effect of ferric transferrin on release of iron from BeWo Chorion carcinoma cells, and suggested that the diferric transferrin first had to be converted to apotransferrin [16]. However, if this were the case here, or if this effect had been due to contamination of the diferric transferrin with apotransferrin (or vice versa), then different potencies for the two forms might have been expected, and this was not observed (Fig. 2A). The effects were not additive, since the addition of iron-transferrin to incubations containing a saturating amount of apotransferrin failed to augment the release (Fig. 2B). A possible explanation for these effects is that the two forms of transferrin are accessing the same intracellular pool of iron, with the apotransferrin accelerating its release by providing a large iron-binding pool to accept iron, and the receptor-mediated endocytosis of diferric transferrin 'chasing' it out by obliging the cell to take up unlabelled iron. The concentration-dependent effect of apotransferrin indicates that it is acting as more than simply a passive acceptor of iron in the extracellular medium. In previous work we found no evidence of receptors specific for apotransferrin on hepatocytes [3]; apotransferrin is nevertheless able to bind to the standard transferrin receptor [17] and so it is possible that this receptor facilitates transferrin binding to iron either at the cell surface or within the cell.

The mechanism of iron release from liver cells has long been thought to involve the oxidation of ferrous to ferric iron, and much of the basis for this comes from the effect of the ferroxidase ceruloplasmin on the release. Very early work showed that ceruloplasmin augmented the release of iron from the perfused rat liver to apotransferrin [2]. A more recent study confirms that a similar mechanism is important during iron absorption from the gut, and in particular for the release at the contraluminal side into the portal blood. Here, iron was found to be in the ferrous state and ceruloplasmin accelerated its release into the portal blood by catalyzing the oxidation of ferrous iron by means of its high Fe(II) : oxygen oxido-reductase activity [18]. We have now shown that this ceruloplasmin-mediated acceleration of iron release occurs directly on HepG2 liver cells. The action of ceruloplasmin was noted only in the presence of high concentration transferrin (≥ 200 µg/ml; Fig. 3). It is possible that at low transferrin concentrations, the rate of spontaneous oxidation is sufficient to manage the corresponding slow iron release rate; at higher release rates, however, an exogenous

ferroxidase activity, such as that provided by ceruloplasmin, is required.

The action of ceruloplasmin on apotransferrin-mediated iron release is influenced not only by the apotransferrin concentration (as shown above), but also by the ambient oxygen concentration. When the iron release under low ambient oxygen was studied, ceruloplasmin augmented the rate induced by apotransferrin (Table 1); unlike the effect in ambient oxygen, this increase occurred even at the low apotransferrin concentration of 20 µg/ml. These experiments suggest that the ferroxidase activity of ceruloplasmin may only become important when iron release is occurring rapidly or when the amount of oxygen is limited. It is possible that, when the rate of release is low, released iron may be oxidized by dissolved molecular oxygen, but when the rate of release is higher or the supply of oxygen is limited a ferroxidase in the form of ceruloplasmin is required. Culture medium has an oxygen concentration of about 216 µM while blood oxygen is about 53–120 µM [19]. Deep within the liver it may well be even lower [20] and so ceruloplasmin-catalyzed oxidation of iron for binding to apotransferrin [19] may be critical to prevent a rise in free iron under these low oxygen conditions.

Clinical observations support the involvement of ceruloplasmin in iron balance. Recently, two separate clinical studies have reported genetically confirmed ceruloplasmin mutations resulting in truncated and thus dysfunctional proteins. In both cases, this was associated with systemic and pathological iron deposition, primarily in the liver but also elsewhere [21,22]. In a third report, not only were hepatic iron deposition and low serum iron accompanied by aceruloplasminemia, but intravenous administration of ceruloplasmin considerably increased iron mobilization [1].

In man, as other species, ceruloplasmin is a known acute phase protein, rising in conditions of infection and inflammation, which are known to pose a disruptive threat to intracellular and extracellular iron balance [23]. Our results indicate that ceruloplasmin has a function as a major modulator of hepatocellular iron release, and thus of systemic iron homeostasis.

Acknowledgements: This work was supported by the Arthritis and Rheumatism Council.

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