TRANSFERRIN PROTEIN AND IRON UPTAKE BY CULTURED RAT FIBROBLASTS

Jean-Noël OCTAVE, Yves-Jacques SCHNEIDER, Pierre HOFFMANN, André TROUET and Robert R. CRICHTON

Université Catholique de Louvain, Unité de Biochimie, 1, Place Louis Pasteur, B-1348 Louvain-la-Neuve, Belgium, Laboratoire de Chimie Physiologique, 75, Avenue Hippocrate, B-1200 Bruxelles and *International Institute of Cellular and Molecular Pathology, 75, Avenue Hippocrate, B-1200 Bruxelles, Belgium*

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1. Introduction

Serum iron is transported bound to transferrin. The transferrin molecule can bind a maximum of two atoms of iron in the ferric form with concomitant binding of carbonate or bicarbonate ion (reviewed in [1]). The iron bound to transferrin can be taken up by many different cell types; inside the cell the iron is incorporated into the storage form, ferritin, and may also be used for the synthesis of iron-containing haem and non-haem enzymes. From studies, mostly carried out with reticulocytes, it has been established that subsequent to the binding of transferrin to specific receptors on the plasma membrane the transferrin molecule is internalised by endocytosis and the iron is released [2,3]. The precise mechanism and site of iron release remains unclear, and it is at present not established whether interiorisation of the transferrin molecule precedes or is a subsequent event to iron release. It has been proposed [4] that subsequent to endocytosis of the transferrin–receptor complex iron would be released in endocytic vacuoles while the iron-depleted protein would be returned to the extracellular medium. In view of the lability of transferrin iron at acid pH values another mechanism can be envisaged. Studies on the fate of antibodies directed against the plasma membrane of cultured rat fibroblasts lead [5] to the conclusion that plasma membrane fragments, after interiorisation, gain access to lysosomes and could thereafter be recycled back to the cell surface. By analogy with these results we propose that the transferrin molecule, after binding and internalisation in endocytic vacuoles, gains access to lysosomes where its iron is released, and the transferrin molecule is returned to the cell surface and discharged into the extracellular medium. Results compatible with this hypothesis have been obtained in experiments using rat fibroblasts in culture [6].

We report here on experiments which show that cultured rat fibroblasts accumulate transferrin iron, and that a number of drugs which affect endocytosis and lysosomal function or which chelate free iron (but not directly transferrin or ferritin iron) greatly reduce iron uptake from transferrin by fibroblasts without significantly affecting accumulation of transferrin protein.

2. Materials and methods

Rat serum transferrin was purified by a modification of the method in [7] using $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography, chromatography on Sepharose–protein A and a final chromatography on CM-cellulose [6]. Transferrin was labelled with ^3H by reductive methylation [8] using sodium [^{3}H]borohydride and the apotransferrin was labelled with ^{59}Fe as ferric citrate in order to have a specific activity close to that for ^3H [6]. The transferrin was subsequently saturated with non-radioactive iron to give a A_{465}/A_{280} ratio of 0.046 [9]. Rat embryo fibroblasts were prepared and cultivated as in [10]. The culture medium was supplemented with 10% newborn calf serum (NBCS). The cells were incubated at 37°C with labelled transferrin at 100 μg/ml final conc. and after incubation the medium was removed, the cells washed 4 times with phosphate-buffered saline (PBS), once with culture medium

containing 10% NBCS, twice with PBS to detach seric protein simply adsorbed, and solubilised with 1% (w/v) sodium deoxycholate adjusted to pH 11.3 with NaOH. The radioactivity accumulated by the cells was measured using an Aqualuma cocktail in a Packard liquid scintillation counter. All samples were corrected for quenching. Cell protein was determined by the Lowry method [11]. The results are expressed as dpm/mg cell protein. The drugs were employed at the final concentrations given in section 3 (all of which were sub-toxic for the cells) throughout the incubation.

3. Results and discussion

That rat fibroblasts in culture accumulate transferrin iron and transferrin protein is apparent from fig.1. At 37°C iron uptake proceeds linearly over the entire period of incubation studied (24 h). In contrast, accumulation of ^{3}H-labelled material increases linearly up to ~6 h, then reaches a plateau. Since the specific activities of ^{3}H and ^{59}Fe are very similar at the start of the incubation, these results establish that fibroblasts accumulate considerably more ^{59}Fe than ^{3}H. After 24 h much of the ^{59}Fe is associated with cytosol ferritin [6] and the difference between the accumulation of iron and protein cannot be explained by the digestion of the transferrin and the release of degradation products into the extracellular medium [6].

Desferrioxamine B, 2,3-dihydroxybenzoate and 2,2′-bipyridyl are all iron chelators. At final concentrations of 50 μM, 1 mM and 50 μM, respectively, they all greatly reduce uptake of ^{59}Fe without a correspondingly important effect on ^{3}H accumulation (fig.2). For all three chelators iron accumulation proceeds at a rate which is not markedly below control values for the first 4–6 h and then slows down considerably. None of these three drugs is an effective chelator of transferrin or of ferritin iron [12,13]. The possible mode of action is discussed further below.

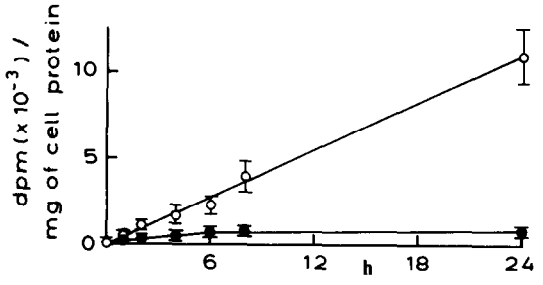

Fig.1. Kinetics of accumulation of ^{59}Fe (○) and ^{3}H (●) -labelled material by fibroblasts. Cells (~300 μg protein) were incubated in 7 cm² plastic Petri dishes, in 0.3 ml culture medium supplemented with 10% calf serum and containing 100 μg/ml of ^{59}Fe-loaded, ^{3}H-labelled transferrin for various times at 37°C. Mean results of 12 expt ± SD.

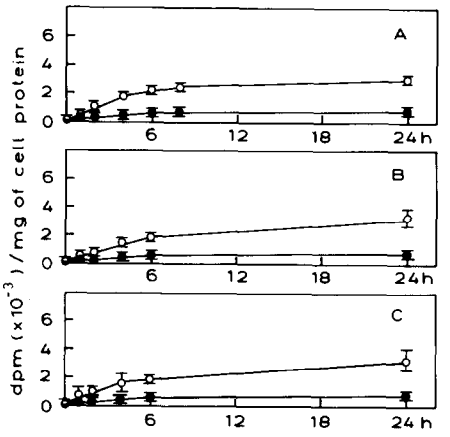

Fig.2. Kinetics of accumulation of ^{59}Fe (○) and ^{3}H (●) -labelled material by fibroblasts. Cells (~300 μg protein) were incubated in 7 cm² Petri dishes, in 0.3 ml culture medium containing 100 μg/ml ^{59}Fe-loaded, ^{3}H-labelled transferrin and 50 μM 2,2′-bipyridyl (A), 1 mM 2,3-dihydroxybenzoate (B), or 50 μM desferrioxamine B (C), for various times at 37°C. Mean results of 3 expt ± SD.

Chloroquine and methylamine are weak bases which accumulate in lysosomes [14]. Their effects on iron and ^{3}H accumulation in fibroblasts incubated with doubly-labelled transferrin are illustrated in fig.3. At 10 μM chloroquine has no effect either on ^{59}Fe or on ^{3}H accumulation. At 100 μM chloroquine substantially reduces the rate of ^{59}Fe accumulation, without any significant effect on ^{3}H uptake. Interestingly, in contrast to the iron chelators used (fig.2) ^{59}Fe uptake continues in a linear fashion over 24 h, but the rate of iron accumulation is reduced to ~50% of control values. With methylamine at 10 mM final conc. a similar effect is observed. Iron uptake is linear over the 24 h incubation period, but the rate of accumulation of ^{59}Fe is substantially reduced compared to control values.

As pointed out in section 1 the precise site of trans-

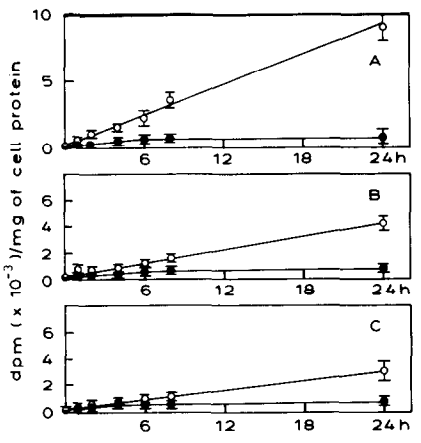

Fig.3. Kinetics of accumulation of ^{59}Fe (○) and ^{3}H (●) -labelled material by fibroblasts. Cells (~300 μg proteins) were incubated in 7 cm^2 Petri dishes, in 0.3 ml culture medium containing 100 μg/ml ^{59}Fe-loaded, ^{3}H-labelled transferrin and 10 μM chloroquine (A), 100 μM chloroquine (B) or 10 mM methylamine (C) for various times at 37°C. Mean results of 3 expt ± SD.

ferrin iron release inside the cell is not known, but we may suppose that either iron is released at the cell membrane subsequent to transferrin binding, or is released after interiorisation of the transferrin–receptor complex as an endocytic vacuole, possibly after fusion of the endocytic vacuole with a lysosome. In terms of the second model, transferrin depleted of iron would subsequently be returned to the plasma membrane and released into the extracellular medium [5,6].

In cultured mouse peritoneal macrophages, 10 mM methylamine and 100 μM chloroquine increase the lysosomal pH from 4.8–6.2 [15]. If iron is released from transferrin subsequent to its binding and interiorisation, we could explain the action of these two drugs by assuming that, as in macrophages, they increase the lysosomal pH and thereby reduce the amount of iron released from transferrin. On the other hand it has been shown that 30 mM methylamine, but not 100 μM chloroquine, inhibits the clustering of α-2 macroglobulin and of epidermal growth factor at the cell surface of fibroblasts [16], a step which appears to be preliminary to their endocytosis. In addition we have observed that these two drugs have a considerable effect on the intracellular processing of several macromolecules, but do not affect their binding to plasma membrane (Y.-J.S., A.T., in preparation). These data suggest therefore that, in addition to increasing the lysosomal pH, methylamine and chloroquine could affect the endocytosis of transferrin.

Desferrioxamine B and 2,3-dihydroxybenzoate, as well as 2,2′-bipyridyl not only reduce iron uptake, but diminish the rate of iron accumulation. We suggest that these molecules penetrate the plasma membrane and are able to compete with ferritin, or other intracellular acceptors for the iron once it has been released from transferrin. Since the resulting iron chelators are quite stable and of low molecular weight, they may eventually be released from the cells into the extracellular medium, thus resulting in a net reduction in iron uptake by the cells.

Bipyridyl may exert its effect on iron accumulation by a combination of the two effects discussed above. By analogy with methylamine and chloroquine, it is a weak base and, if it accumulates inside the lysosomes it could increase their pH. It is also an iron chelator, and thus could also chelate transferrin iron released in lysosomes. At the pH values found in lysosomes bipyridyl is still an extremely effective iron chelator.

These preliminary studies establish that cultured rat fibroblasts are a potentially useful system for the study of transferrin iron uptake, particularly since cell fractionation procedures are well developed [10]. We have found that not only do they accumulate transferrin iron but that drugs, which effect endocytosis and lysosomal function, and also iron chelators, inhibit transferrin iron accumulation by the cells.

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