Inhibition of endosome fusion in primary hepatocytes prevents asialoglycoprotein degradation but not uptake of transferrin iron demonstrating that intracellular iron release occurs from early endosomes

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Abstract A comparison of the effects of inhibitors of membrane fusion on the uptake of asialoglycoprotein and transferrin by primary rat hepatocytes was made. This showed that while high potassium medium inhibited the degradation but not the uptake of asialoorosomucoid, both transferrin endocytosis and iron delivery to the cells were unaffected. This difference between the two pathways was also observed with an inhibitor of phospholipase A2, bromophenacyl bromide. With the latter, it was found that the asialoglycoproteins failed to traverse from a low-density to a high-density intracellular compartment, implying a role for phospholipase A2 in the trafficking of asialoglycoprotein receptor but not that for transferrin or iron. This demonstrates that, after its release from transferrin, iron is transported to the cytoplasm directly from the early endosome without the need for fusion of the iron-containing vesicle with a lysosome.

Key words: Endocytosis; Asialoglycoprotein receptor; Endosome fusion; Phospholipase A2; Transferrin; Bromophenacyl bromide; Hepatocyte; Iron

1. Introduction

The asialoglycoprotein receptor, present on the surface of primary hepatocytes, functions in the clearance of desialylated proteins from the plasma. It recognises, and binds, exposed galactose residues and target proteins are endocytosed and finally degraded in the lysosomes [1]. Early events in the uptake of asialoglycoproteins have been fairly well characterised and these are common to another major endocytic pathway, that of transferrin [2]. Both are dependent on clathrin-assisted invagination of the plasma membrane, followed by accumulation in an intracellular sorting compartment (CURL). Following this, it is thought that the pathways for the ligands may diverge, while the receptors are recycled, probably by the same mechanism. In a previous study, we compared the fate of transferrin and asialotransferrin when taken up via the transferrin receptor and the asialoglycoprotein receptor [3]. This showed that transferrin could be either degraded or recycled depending on which receptor it bound to initially. During that study we also observed that iron could be delivered to the cell from transferrin taken up via the asialoglycoprotein receptor, which suggests that both iron and asialoglycoprotein delivery could be dependent on their delivery to other compartments downstream of CURL. Two mammalian cell transmembrane metal transporters have recently been described. Nramp1 [4] is restricted to lysosomal membranes while the closely related Nramp2 is found in endosomes [5]. The question arises whether under normal conditions, following release of iron from transferrin in hepatocytes, the metal is immediately released from the endosome or follows an endocytic pathway towards the multi-vesicular vesicles/lysosomes before being released from the membrane-bound compartment to the cytoplasm.

A number of studies have made use of metabolic inhibitors to define the biochemical events in protein endocytic pathways. For instance, incubation of cells in the absence of sodium ions inhibited the delivery of the asialoprotein to the lysosomes while the uptake process was not [6,7]. Similarly, inhibitors of phospholipase A2 have been shown to interfere [8]. Because these inhibitors may act at points in the pathways where transferrin and asialoglycoprotein uptake diverge we wished to compare their activities on the two pathways to uncover any differences. We found that neither inhibitor prevented uptake of transferrin or iron while asialoglycoprotein accumulated in a light vesicle in an undegraded form, suggesting that the pathways diverge prior to the fusion of endosomes and that the iron uptake pathway is independent of this event implying that iron is released from an early endosome.

2. Materials and methods

2.1. Proteins

Rat transferrin was purified from pooled serum using ion exchange chromatography as described before [9]. Human orosomucoid (Sigma, Poole, UK) was desialylated as previously described [3] and radiolabelled with ^{125}I (NaI from Amersham, Bucks, UK) using lactoperoxidase-Sepharose [10]. Transferrin was made iron-free by dialysis against citrate buffer, labelled to 98% saturation with $^{59}FeCl_3$ (Amersham) and then labelled with ^{125}I [11]. Unbound ^{125}I and ^{59}Fe-citrate were removed by passage of the protein through Dowex 1-X8 anion exchange resin, equilibrated with 25 mM HEPES, to yield labelled proteins in which the ^{125}I label was 97% precipitable with 5% trichloroacetic acid (TCA).

2.2. Hepatocytes

Male Wistar rats (250–300 g) were anaesthetised with thiopental and the livers were perfused with collagenase as described before [12,13]. Cells were suspended in minimal essential medium (MEM) (Life Technologies, Fife, UK) with 1% bovine serum albumin (BSA, fraction V, Sigma). At least 95% of the cells excluded trypan blue.

2.3. Incubation procedures

Hepatocytes ($7–12\times10^6$ cells/ml in MEM) were incubated at 37°C in a volume of 2.5 ml in a shaking water bath under 5% CO_2/air.

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Bromophenacyl bromide (BPB), dissolved in dimethyl sulphoxide, was added to give final concentrations of 100–1000 μM. A series of experiments showed that a concentration of 500 μM gave maximum effects without compromising cell viability and so this was used throughout the experiments described. Control incubations, containing just dimethyl sulphoxide, were performed in parallel and no effects of the solvent were observed. In experiments with high-potassium buffer [6] the cells were washed once in this buffer and then suspended, at the same cell density as those in MEM, in K^+ buffer containing 1% BSA and incubated under air. After 20 min, cells were cooled rapidly on ice and ^{125}I-labelled asialoorosomucoid was added to give a final concentration of 5 μg/ml. After 30 min cells were washed three times in ice-cold MEM, re-suspended in pre-warmed medium containing the inhibitor and the incubation continued at 37°C. For experiments with transferrin, cells were taken through the same procedure in the absence of labelled protein and ^{59}Fe-^{125}I-labelled diferric rat transferrin (20 μg/ml) was added when the cells were re-suspended in warm medium. Samples (0.3 ml) were taken at intervals into ice-cold phosphate-buffered saline (PBS) containing EGTA (5 mM) to release surface-bound asialoorosomucoid into the medium, and the cells were centrifuged at $400\times g_{(av)}$ for 3 min. The supernatants were collected and those containing asialoorosomucoid were made 10% with TCA and left overnight on ice. The cells were washed a further three times and those with asialoorosomucoid were suspended in 10% TCA. The TCA suspensions were centrifuged at $900\times g_{(av)}$ and the pellets and supernatants collected. These, together with the cell pellets containing ^{59}Fe-^{125}I-labelled transferrin, were then counted in a dual channel LKB Compugamma gamma-counter.

2.4. Intracellular distribution of ligands

Hepatocytes (5×10^6/ml) were treated as above but after incubation at 37°C samples were taken into ice-cold PBS, washed twice in PBS, once in 0.25 M sucrose/10 mM EDTA/10 mM Tris–HCl pH 7.4, and then re-suspended in this sucrose buffer. They were then homogenised, on ice, with 40 strokes of a Dounce homogeniser and applied to a Percoll (Pharmacia, St Albans, UK) gradient as before [14]. Following centrifugation, fractions were removed by piercing the bottom of the tubes for counting of radioactivity and assays of marker enzymes 5′-nucleotidase and acid phosphatase [14].

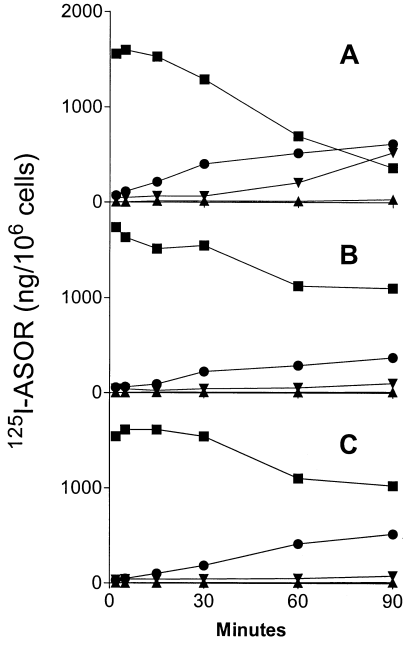

Fig. 1. Effect of BPB and high potassium on hepatocyte uptake and degradation of asialoorosomucoid. Rat hepatocytes, with surface-bound ^{125}I-asialoorosomucoid, were incubated at 37°C and sampled at times into PBS with EGTA (5 mM). Label associated with the cells, which was TCA-soluble (▲) or insoluble (●) and label in the medium which was TCA-soluble (▼) or insoluble (■) was determined. A: Cells in medium alone. B: Cells in medium with high potassium. C: Cells in medium with BPB. These experiments were repeated on at least six occasions with similar results.

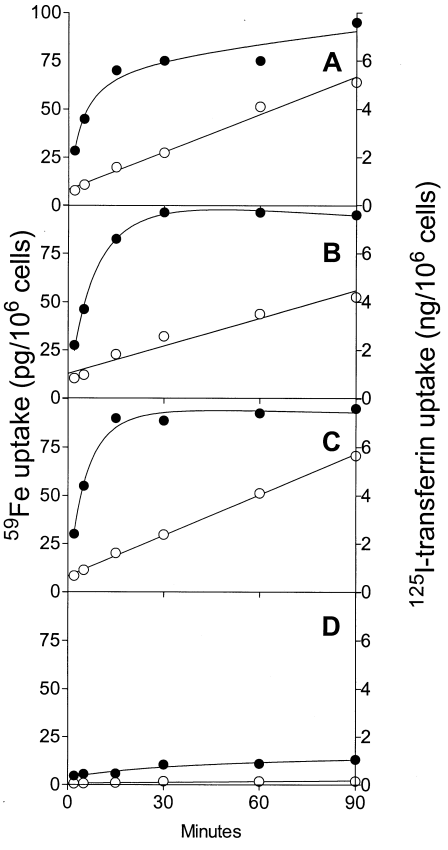

Fig. 2. Effect of BPB and high potassium on hepatocyte uptake of transferrin and iron. Rat hepatocytes were incubated with ^{125}I-^{59}Fe-labelled rat transferrin and sampled over time to monitor iron (○) and transferrin (●) uptake. A: Cells in medium alone. B: Cells in medium with high potassium. C: Cells in medium with BPB.

3. Results

To investigate the uptake of asialoorosomucoid by hepatocytes the cells were first surface-labelled with labelled proteins in the cold, washed and then warmed rapidly to 37°C to allow internalisation of the ligands. As shown in Fig. 1A there was a steady loss of ligand from the surface of the cells as the incubation proceeded which was balanced by the accumulation of intracellular protein. After a lag of 30 min, acid-soluble label began to appear in the medium and this accumulated more rapidly towards the end of the incubation period. There was no evidence for the accumulation of acid-soluble product within the cells.

When the cells were incubated in the high-potassium buffer, although loss of material from the surface was blunted, uptake continued to occur at a substantial rate and this was associated with accumulation of the whole protein in the cells (Fig. 1B). However, under these conditions there was no evidence for any degradation of the protein taken up or any accumulation of degradation products within the cell. Very similar results were obtained when the cells were incubated with the phospholipase A2 inhibitor BPB, and in the experiment shown, at 90 min, degraded protein release was de-

creased by 85% from 522 ng/10^6 cells to 78.5 ng/10^6 cells (Fig. 1C).

In contrast, when the uptake of transferrin and iron was measured under the same conditions neither high-potassium buffer (Fig. 2B) nor BPB (Fig. 2C) had any measurable effect on the uptake of the protein or the metal. It was confirmed that uptake was occurring via the transferrin receptor by performing incubations in the presence of excess unlabelled transferrin and under these conditions, the iron uptake was abolished, as was transferrin uptake (Fig. 2D).

To investigate the intracellular fate of the asialoorosomucoid, cells were incubated as above, then homogenised and intracellular components separated on Percoll gradients during the incubation period. As can be seen in Fig. 3 initially most of the ligand was associated with plasma membrane components as shown by a high content of 5′-nucleotidase (Fig. 3, bottom panel) but as the incubation proceeded protein was lost from this compartment and appeared further down the gradient in denser areas some of which were high in acid phosphatase (Fig. 3, bottom panel). Soluble material was also released from the cells and appeared above the gradient and

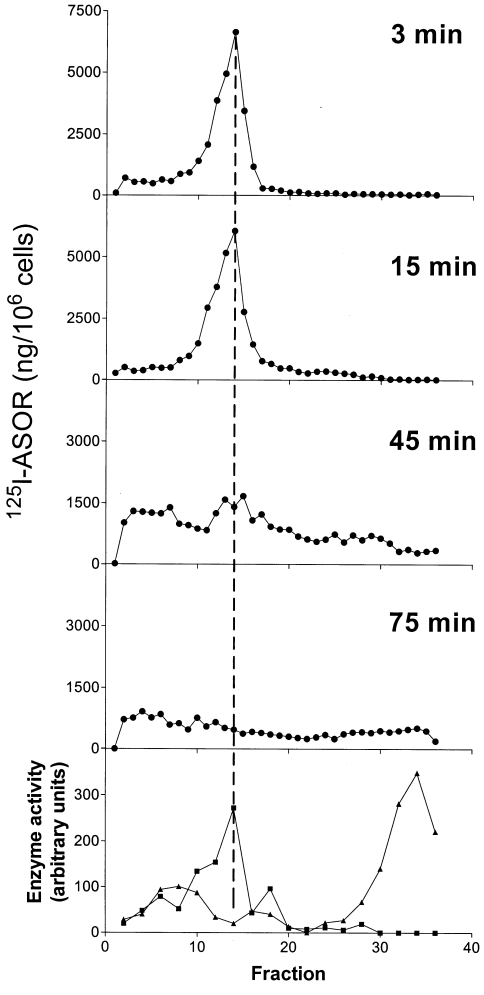

Fig. 3. Distribution of asialoorosomucoid in control hepatocyte intracellular compartments separated on Percoll density gradients. Cells were incubated with ^{125}I-labelled protein, sampled at intervals, washed, homogenised and fractionated on Percoll density gradients. Distribution of marker enzymes 5′-nucleotidase (■) and acid phosphatase (▲) in the gradients was also assessed.

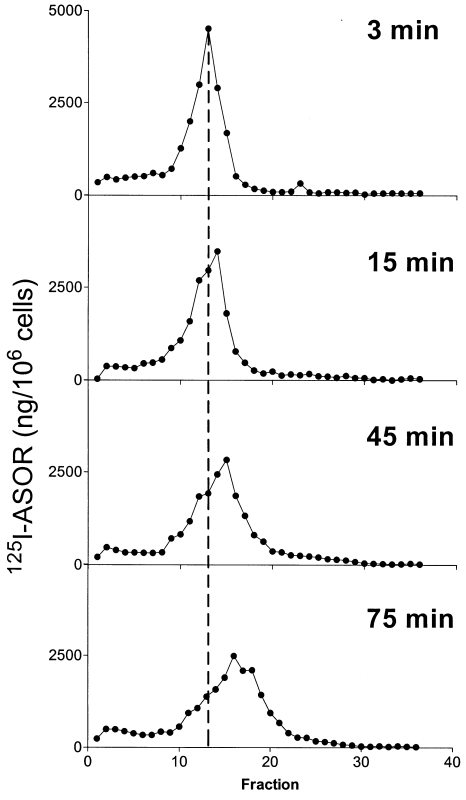

Fig. 4. Distribution of asialoorosomucoid following incubation in BPB in hepatocyte intracellular compartments separated on Percoll density gradients. Cells were incubated as in Fig. 3 but in the presence of BPB.

by 75 min, in this experiment, most of the material had been cleared from the cells. In the presence of BPB, however, even after 75 min incubation the protein was still associated with intracellular compartments and there was no evidence of degradation products being released (Fig. 4). Furthermore, although the protein showed a shift to a higher-density compartment there was no transfer of protein to high-density regions of the gradient where lysosomes would be expected, as shown by the distribution of the acid phosphatase (Fig. 3, bottom panel).

4. Discussion

The inhibitors we have investigated have very different mechanisms of action but they may act at similar points in the endocytic pathway. Barbieri et al. [15] have shown that in high-potassium buffer the fusion of isolated endosomes was inhibited probably through its actions on the rab5 protein. BPB, in contrast, is a potent inhibitor of phospholipase A2 and endosome fusion [8] and results suggest that this enzyme activity is important in fusion of early endosomes rather than more mature later endocytic compartments. However, phospholipase A2 found in reticulocyte endocytic vesicles, which are very active in transferrin endocytosis, is not inhibited by BPB [16]. There are several forms of cellular phospholipase A2 and the high-molecular-weight membrane-associated forms such as that in the reticulocyte vesicle are known not to be inhibited by BPB [17] and would probably not be involved in vesicle fusion. A more likely target is the low-mo-

lecular-weight cytoplasmic phospholipase A2 which is inhibitable by BPB [17]. In isolated vesicle preparations arachidonic acid can reconstitute fusion of vesicles in the presence of inhibited phospholipase A2 [8], suggesting this product of the enzyme controls the fusion process.

We have shown a very distinct difference in the effects of high potassium buffer and BPB on the transferrin and asialoglycoprotein uptake pathways in primary hepatocytes. While the receptors for these two pathways follow very similar endocytic and recycling processes, the ligands, which bind to them, suffer different fates. Asialoglycoproteins are destined for degradation in lysosomes while transferrin is recycled along with its receptor for another round of iron delivery elsewhere. However, it is largely apo-transferrin that is recycled to the surface [18] while its passenger iron atoms are deposited in the cell for incorporation into enzymes or for storage in cytoplasmic ferritin. Thus, iron and asialoglycoproteins are both retained by hepatocytes, but the contrasting effects of the inhibitors on these suggest that their handling by the cell is different.

Inhibition of phospholipase A2 has been shown to prevent plasma membrane invagination during phagocytosis [19] but its lack of inhibition in both asialoorosomucoid and transferrin uptake suggests that there is no requirement for phospholipase A2 activity in clathrin-dependent endocytosis. Asialoorosomucoid, however, accumulated undegraded in a low-density vesicle after endocytosis. This could have been a multi-vesicular vesicle, which are of similar density to early endosomes [2], but, in view of the results described above on the importance of phospholipase A2 in the earlier stages of endocytosis [8], is more likely to have been an early endosome compartment. It is in this compartment that sorting of ligands and receptors takes place [2], and because of the lack of inhibition by BPB and potassium on iron uptake, the results suggest that iron removal from transferrin also occurs here. If this is the case then there is no requirement for fusion of this vesicle with a multi-vesicular vesicle or lysosomes for iron release to occur. Indeed, in previous work we showed that iron could be directly released in an ATP-dependent manner from isolated vesicles in vitro [20]. However, inhibition of lysosome function has been shown to prevent ferritin iron recycling [21,22], which suggests that the later vesicles are capable of releasing iron to the cytoplasmic acceptors. Lysosomes in macrophages are known to express the metal transport protein Nramp1, which is capable of transporting iron [4]. However, it is not clear in which direction this protein directs the iron, since there are reports of it releasing iron from lysosomes to the cytoplasm [23–25] and of promoting uptake of cytoplasmic iron into phagosomes [26]. Early endosomes do not express Nramp1 [5] but instead have a related protein, Nramp2 [27], which co-localises with transferrin in haemopoietic cell precursors [5]. However, the relative expression of these protein in the hepatocyte has not been reported, but since these cells are the site for the body's major ferritin iron store, which is rapidly mobilised when needed [28–31], it would seem that both proteins are likely to be expressed by these cells. The results presented in this paper suggest that even if it is expressed, Nramp1 is unlikely to play a role in transferrin-iron uptake by hepatocytes, since fusion of the endosome with the late vesicles/lysosomes likely to express this protein was not involved in uptake of iron. Our previous work showed that interference with the iron uptake process can lead to iron transferrin being returned to the surface without iron removal [18] and so iron transfer from this early endosome compartment is likely to be the rate-limiting step in iron uptake.

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