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Receptor-mediated uptake of ferritin-bound iron by human intestinal Caco-2 cells☆

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Abstract

Ferritin (Ft) is a large iron (Fe)-binding protein (~450 kDa) that is found in plant and animal cells and can sequester up to 4500 Fe atoms per Ft molecule. Our previous studies on intestinal Caco-2 cells have shown that dietary factors affect the uptake of Fe from Ft in a manner different from that of Fe from $FeSO_4$, suggesting a different mechanism for cellular uptake. The objective of this study was to determine the mechanism for Ft–Fe uptake using Caco-2 cells. Binding of ^{59}Fe-labeled Ft at 4°C showed saturable kinetics, and Scatchard analysis resulted in a K_d of 1.6 μM, strongly indicating a receptor-mediated process. Competitive binding studies with excess unlabelled Ft significantly reduced binding, and uptake studies at 37°C showed saturation after 4 h. Enhancing and blocking endocytosis using Mas-7 (a G-protein activator) and hypertonic medium (0.5 M sucrose), respectively, demonstrated that Ft–Fe uptake by Mas-7-treated cells was 140% of control cells, whereas sucrose treatment resulted in a statistically significant reduction in Ft–Fe uptake by 70% as compared to controls. Inhibition of macropinocytosis with 5-(*N*,*N*-dimethyl)-amiloride (Na^+/H^+ antiport blocker) resulted in a decrease (by ~20%) in Ft–Fe uptake at high concentrations of Ft, suggesting that enterocytes can use more than one Ft uptake mechanism in a concentration-dependent manner. These results suggest that Ft uptake by enterocytes is carried out via endocytosis when Ft levels are within a physiological range, whereas Ft at higher concentrations may be absorbed using the additional mechanism of macropinocytosis.

Keywords: Ferritin; Ferritin receptor; Iron; Iron absorption; Caco-2 cells

1. Introduction

Ferritin (Ft) is an iron (Fe)-binding protein with very high capacity to bind Fe; up to 4500 atoms of Fe can be bound to each Ft molecule [1]. Since several plants used as staple foods express Ft [2–4], it has been proposed that Ft could be used for biofortification with Fe [5]. While the natural content of Ft in staple foods like rice and legumes is relatively low, concentrations can be increased considerably either by conventional plant breeding methods, selecting for high Ft varieties, or by genetic modification approaches, overexpressing the gene for Ft [6–10]. If proven successful, this would provide a sustainable method for Fe fortification. It is important, however, that the Fe in Ft is in a bioavailable form so that populations can benefit from this Fe source when it is part of their regular diet.

The bioavailability of Fe from Ft has been assessed in several recent human studies [11–13]. Some early studies showed poor bioavailability of this form of Fe, most likely because inappropriate labeling techniques were used [14–16]. In some studies, animal Ft induced by various methods was used and it is now known that such Ft is not representative for "normal" Ft sources. Extrinsic labeling by adding radioiron directly to Ft was used in some studies, but this added isotope does not equilibrate with the insoluble Fe inside the Ft core. Using an appropriate and validated extrinsic labeling technique, we have recently shown that human subjects absorb Fe from both animal and plant Ft well [12,13] and to an extent similar to that from $FeSO_4$. These results suggest that Ft may be a useful vehicle for biofortification with Fe.

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Fe deficiency and anemia in developing countries are usually due to a combination of low Fe intake and diets containing factors inhibiting Fe absorption. Although Fe intake may be increased by consumption of plant diets with increased Ft, it is important to assess the effects of dietary factors on Fe absorption from this source. We have recently assessed this in vitro [17], using Caco-2 cells, a well-established human intestinal cell line that, in culture, differentiates into enterocyte-like cells. This cell line has been utilized for assessment of Fe bioavailability from various sources [18–25]. Using radiolabeled Ft and Caco-2 cells in monolayers, we found that phytate, tannic acid and calcium, which are known to inhibit absorption of ferrous Fe [18,21,25–30], and ascorbic acid, which enhances Fe absorption [19,25,31], had significantly less pronounced effect on Fe uptake from Ft as compared to that observed for Fe uptake from $FeSO_4$ [17]. This strongly suggests that Fe is taken up from Ft via a mechanism different from that for ferrous Fe, which is known to occur via divalent metal transporter-1 (DMT-1) [32,33]. Our in vitro digestion experiments suggested that Ft may survive proteolytic degradation by pepsin and pancreatic enzymes under conditions similar to those of the human gastrointestinal tract.

We have shown in human studies that Fe absorption is similar from horse spleen Ft with plant-type mineral and animal-type mineral and from ferrous sulfate [12]. Further, we have shown in human studies that Fe absorption from soybean Ft was similar to that from $FeSO_4$ [13]. In this study, we explored alternative pathways for Fe uptake using animal Ft and the Caco-2 cell model.

2. Materials and methods

2.1. Labeling of Ft with ^{59}Fe

Animal Ft (horse spleen) was purchased from Calzyme Laboratories (San Luis Obispo, CA) and then dialyzed to remove the Fe core following the protocol previously described [12]. Briefly, Fe was removed by thioglycolic acid reduction and dialysis. Fe content of the dialyzed Ft was measured using atomic absorption spectroscopy. The apoprotein shell was then reconstituted using ferrous Fe with a radioactive Fe tracer. Radioactive Fe (^{59}Fe as $FeSO_4$; specific activity, 27.7 mCi/mg) was purchased from Perkin Elmer (Boston, MA). Since our primary objective was to study Fe uptake from Ft, we preferred this method of labeling, particularly as several previous studies have shown validity problems with using ^{125}I labeling of Ft [34,35]. The radiolabel was incorporated to ~90%. Prior to the administration of this radioactive Ft to cells, it was subjected to several buffer exchanges using Centricon filter tubes (30 kDa molecular weight cutoff) to ensure removal of loosely bound surface radioactivity. Radioactivity associated with the filtered fractions was measured using a gamma counter (Gamma 8500, Beckman, Irvine, CA) until the nonspecific radioactivity was close to background values. Thus, the nonspecific radioactivity removal was over 99.9% efficient.

2.2. Cell culture

Caco-2 cells (American Type Tissue Culture Collection, Rockville, MD) were seeded (225,000 cells/cm^2) onto cell-culture-treated polystyrene plates (used between passages 30 and 40) and cultured in Minimal Essential Medium (MEM) (Invitrogen Life Sciences, Rockville, MD) containing 10% fetal bovine serum (Sigma) and antibiotics (penicillin, 10 U/ml; streptomycin, 1 mg/ml) at 37°C with 5% carbon dioxide. Cell protein was assessed using the Bradford assay [36]. Data are expressed as picomoles of Fe per microgram of cell protein.

2.3. Ft binding and uptake

2.3.1. Binding studies

To determine the cell stage that allowed for maximal Ft binding, we treated cells on polystyrene plates with ^{59}Fe–Ft (1 μM) in serum-free medium for 8 h at 4°C at preconfluent, confluent, 4 days postconfluent and 7 days postconfluent stages. Results from this pilot study determined the optimum treatment stage for subsequent experiments. In order to demonstrate saturable binding kinetics, we treated cells with ^{59}Fe–Ft (0.1–8 μM) in serum-free medium for 8 h at 4°C. In a competitive binding experiment, cells were coincubated with increasing concentrations of unlabeled Ft (0.5–8 μM) to determine binding specificity.

2.3.2. Uptake studies

Cells on polystyrene plates were treated with fixed (1 μM ^{59}Fe–Ft) and increasing concentrations (0.1–8 μM ^{59}Fe–Ft) in serum-free medium for 16 and 1 h, respectively, at 37°C. Medium was removed and cells were washed extensively with cold phosphate-buffered saline (PBS). Cell-associated radioactivity was quantified in the gamma counter.

2.4. Stimulation of endocytosis

Cells grown on polystyrene plates were treated with 50 μM Mas-7 (Sigma-Aldrich), a G-protein activator and a highly potent analog of mastoparan [37,38], for 30 min at 37°C in PBS containing 1 μM ^{59}Fe–Ft and then washed three times with ice-cold PBS to remove any loosely bound radioactivity. Exofacially bound Ft was removed by a brief acid wash (0.15 M NaCl, pH 3.0, for 30 s, on ice), and cellular radioactivity was quantified in the gamma counter. Endosome labeling using Sulfo-Link (sulfo-*N*-hydroxy-succinimidobiotin) (EZ-Link Sulfo-NHS-Biotin Reagents, Pierce Biotechnology, Rockford, IL) was used as positive control to assess endocytosis via confocal microscopy [39]. This method was used to visually detect an increase in cellular uptake of ^{59}Fe–Ft upon incubation with Mas-7, due to lack of a good control for quantitative detection. Cells were biotinylated at the apical membrane by incubation