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Alternative pathways for absorption of iron from foods*

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Abstract: Iron is known to be absorbed from foods in two major forms, heme iron and non-heme iron. Iron status as well as dietary factors known to affect iron absorption has limited effect on heme iron absorption, whereas inhibitors and enhancers of iron absorption have pronounced effects on non-heme iron absorption. The enterocyte transporter for non-heme iron, DMT1, is strongly up-regulated during iron deficiency and down-regulated during iron overload. A transporter for heme iron, HCP1, was recently characterized and is present on the apical membrane of enterocytes. Two other pathways for iron absorption have been discovered and may serve to facilitate uptake of iron from two unique iron-binding proteins, lactoferrin and ferritin. Lactoferrin is an iron-binding protein in human milk and known to survive proteolytic digestion. It mediates iron uptake in breast-fed infants through endocytosis via a specific lactoferrin receptor (LfR). Recently, lactoferrin has become popular as a food additive and may enhance iron status in several age groups. Ferritin is present in meat, but also in plants. The ferritin content of plants can be enhanced by conventional breeding or genetic engineering, and thereby increase iron intake of populations consuming plant-based diets. Ferritin is a bioavailable source of iron, as shown in recent human studies. Ferritin can be taken up by intestinal cells via endocytosis, suggesting a receptor-mediated mechanism.

Keywords: food iron; iron; iron absorption; iron nutrition; iron metabolism.

INTRODUCTION

The human body has developed several mechanisms for acquiring iron from the diet. Traditionally, it has been believed that iron is absorbed in two major forms; heme and non-heme iron. Diets in developing countries contain little or no heme-iron, which is similar to vegetarians who consume no meat products. Iron deficiency is very common world-wide, particularly in less developed countries, and is expected to afflict some 1–2 billion people [1]. Since non-heme iron is such a dominant part of the diet—not even in heavy meat-eaters is heme-iron intake more than 15–20 % of total iron intake [2]—it is not surprising that a considerable effort was made to identify the mechanism for non-heme iron absorption, and that this mechanism was unraveled before those behind absorption of other forms of iron.

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ABSORPTION OF NON-HEME IRON

By the use of radioisotopes, it was early found that there is strong homeostatic regulation of absorption of non-heme iron [2]. Thus, iron-deficient subjects absorb considerably more non-heme iron than iron-replete individuals. This up- and down-regulation of absorption is so strong—absorption varies between some 3–70 %—that if individual differences in non-heme iron absorption are not compensated for, it is impossible to assess the impact of other factors affecting non-heme iron absorption, such as dietary components, food preparation methods, and processing.

The divalent metal transporter (DMT)-1 was characterized in 1997 by Gunshin et al. [3]. DMT1 is a transmembrane protein that transports ferrous iron across the apical membrane. There are four different isoforms of DMT1 in the intestinal epithelial cell, due to alternative splicing causing different start sites (exons 1A and 1B) [4]. One of these variants contains an iron-regulatory element (IRE) and this is believed to be the major functional isoform in the small intestine [5]. The comparatively low pH of the proximal duodenum in combination with the acidic microenvironment at the brush border membrane stabilizes iron in the divalent form and provides protons essential for driving iron uptake across the apical membrane of the mucosa [3,6]. The significance of DMT1 in iron uptake has been shown by antibodies toward DMT1 [6,7] as well as targeted disruption of the gene in mice [8], both inhibiting iron absorption. There are spontaneous mutations in the DMT1 gene in both the mouse (*mk/mk* mice) and the rat (Belgrade rat) leading to microcytic anemia [9,10]. Several mutations in the DMT1 gene in humans have been identified [11,12], which also cause microcytic anemia.

Although iron status clearly affects the expression of DMT1, the localization of DMT1 is key to its role in mucosal iron uptake. Studies in human intestinal (Caco-2) cells in culture have shown that DMT1 expression on the apical membrane rapidly decreased in response to excess iron [13]. It was also shown that DMT1 protein was internalized and targeted toward a late endosomal/lysosomal compartment [14]. This is similar to findings in animal models in which a bolus of iron given orally was shown to cause redistribution of DMT1 [15]. We have found that expression of DMT1 is very low during the neonatal period [16,17] and also that it is mislocalized in young rat pups [18], which may be the reason why rat pups fed iron orally by gavage failed to regulate iron absorption [16,17]. Studies on mouse pups showed that DMT1 was primarily found to be localized intracellularly during the nursing period, whereas at weaning it had the appropriate apical orientation [19]. Lactoferrin receptor (LfR), however, was expressed early in life in the mouse, and had the proper apical localization. It is therefore possible that alternative pathways, such as LfR (see below) are responsible for iron uptake during the neonatal/nursing period.

Similar to observations in rat pups, it has been shown that human infants fail to regulate iron absorption at 6 months of age [20]. There was no difference in iron absorption measured by stable isotopes in infants who had been given iron supplements daily between 4–6 months of age and unsupplemented infants. At 9 months of age, however, infants who had been given iron drops daily absorbed significantly less iron than those not given iron, showing that at this age, homeostatic regulation of iron absorption occurs. While mucosal expression and localization of DMT1 has not been described in human infants at various ages, it is likely that the lack of regulation at young age is due to lack in response and/or mis-localization of this transporter. Similar to the mouse, human milk contains lactoferrin, and it is possible that iron uptake instead is mediated by the LfR (see below).

It is known that DMT1 transports iron in its ferrous form (see above). Since iron in an aerobic environment and neutral pH exists in the poorly solubilized, oxidized ferric state, it needs to be reduced prior to absorption. A need for a brush border associated ferric reductase was therefore postulated, and biochemical evidence for such activity was provided for the mouse [21]. Further, McKie et al. [22] identified and characterized such a reductase, duodenal cytochrome b (Dcytb), in mouse intestinal tissue. Although its gene does not have an IRE, its expression is modulated by iron, in that iron-deficient mice have increased expression of Dcytb protein in the proximal intestine (but not other tissues), and its reductase activity is similarly increased. Antibodies against Dctyb were shown to decrease the ferric re-

ductase activity in brush border membranes [22], strongly suggesting that Dcytb is the major reducing enzyme. However, targeted disruption of the gene encoding Dcytb in mice did not result in iron deficiency [23], suggesting that Dcytb is not essential for iron absorption in mice. It is possible, though, that endogenous production of ascorbic acid in the mouse is adequate to reduce ferric iron to its absorbable ferrous form. It is well known that mice absorb ferric iron well, whereas humans absorb this form of iron poorly [2]. Little is known about the expression and activity of Dcytb in humans, but absorption studies indicate that, if present, its activity is low.

ABSORPTION OF HEME-IRON

The mechanism underlying uptake of heme-iron remained an enigma for quite some time. It was evident that this pathway of iron absorption was distinct from that for non-heme iron as it is affected little by dietary inhibitors or enhancers of iron absorption [2]. It was also apparent from isotope studies on human subjects that there is less homeostatic regulation of heme-iron absorption [2]. Studies in Caco-2 cells showed that heme-iron is taken up by a saturable, carrier-mediated process, indicative of a heme transporter [24]. However, despite many attempts to biochemically isolate a putative "heme receptor" from the small intestine, a transporter for heme-iron was not characterized until 2005. The heme-carrier protein 1 (HCP1) was cloned and characterized from mouse duodenum by Shayegi et al. [25] and was found to be localized at the apical membrane. The HCP1 gene was found to encode a protein of 459 amino acids (molecular weight ~50 kDa) with nine predicted transmembrane regions. The human gene encodes 446 amino acids, and the protein is highly conserved among species. HCP1 transports iron bound to heme from the gut lumen into duodenal enterocytes, and iron is then released by heme oxygenase intracellularly. Considerable homology (~75 %) to metal-tetracycline transporters was found, including both transmembrane and non-transmembrane regions. The authors speculated that this may be due to similarities in structure between heme and metal-tetracycline complexes [25]. That HCP1 in fact transports heme was shown by transfection of the gene into oocytes; heme-iron uptake increased ~2.5-fold. Further, incubating mouse duodenal sacs with antibody toward HCP1 blocked heme-iron uptake significantly, showing an active role in this process. Interestingly, no expression of HCP1 was found in mouse duodenum prior to weaning, which would be consistent with the fact that milk contains no heme-iron. Results from iron-deficient and -loaded mice showed no effect on HCP1 gene expression. Further, human Caco-2 cells that were depleted of iron by chelation showed no effect on HCP1 expression or heme uptake [26]. There was no expression of HCP1 in the ileum, which is consistent with in vivo data on heme-iron absorption in various parts of the small intestine [27]. In control animals, HCP1 protein was localized both to the apical membrane and the cytoplasm, whereas it was almost exclusively localized to the apical membrane in iron-deficient mice [25]. Iron-loading, however, resulted in HCP1 being in the cytoplasm. These results strongly suggest a post-translational mechanism for regulating heme-iron uptake during changes in iron status, which is known to occur in human subjects although the magnitude of adaptation is much lower than that for non-heme iron absorption [28]. Our knowledge about HCP1 in humans and its quantitative significance in heme-iron uptake is still very limited, and no mutations in the human HCP1 gene have yet been described.

ABSORPTION OF IRON FROM LACTOFERRIN

A major form of dietary iron for breast-fed infants is lactoferrin [29]. Breast milk contains high concentrations of lactoferrin, and each lactoferrin molecule can bind two ferric iron ions. Early radioisotope studies showed high bioavailability of iron from breast milk [30], and since intestinal biopsies were found to bind lactoferrin [31] it was suggested that lactoferrin facilitates iron uptake by the intestinal mucosa [32,33]. Such a scenario was possible as intact human lactoferrin is found in significant quantities in the stool of exclusively breast-fed infants, at least up to four months of age [34]. Recombinant human lactoferrin was also shown to resist digestion in vitro [35]. A receptor for lactoferrin was iso-

lated and characterized [36], and it was subsequently cloned in the human [37], mouse [38], and pig [39], all species with milk having high concentrations of lactoferrin. The LfR is a homo-trimer of about 110 kDa in molecular weight, and it is glycosylated [37]. Interestingly, the LfR has also independently been characterized as a lectin (called intelectin) [40]. This intelectin (= LfR) has been postulated to act as a bacterial scavenger, and thus possibly involved in the defense against infection. Further studies are needed on these dual functions of the LfR. LfRs have been reported on several cell types [41], but these receptors have not been cloned and it is not certain if they are similar to the intestinal LfR. The intestinal LfR has been shown by confocal microscopy to mediate the uptake of lactoferrin into the cell [42]. Recent studies suggest that the receptor may also bind bovine lactoferrin [43], but it is not yet known whether this form of lactoferrin is internalized and reaches the nucleus.

Expression of the LfR is very high in late fetal and infant small intestine, while it is lower in adult intestinal tissue [37]. Interestingly, the expression is similarly high in duodenum, jejunum, and ileum [39], possibly allowing lactoferrin-iron uptake throughout the small intestine, whereas the uptake of ferrous iron (mediated by DMT-1) only occurs in the duodenum. This may be one reason why iron absorption is lower from infant formula (where iron is added as ferrous sulfate) than from breast milk [44]. Iron is well absorbed from lactoferrin in human adults also [45], but it is not known whether it is absorbed from intact lactoferrin or in free form from lactoferrin digested by the more efficient adult gastrointestinal tract. Several recent studies have shown a positive effect of bovine lactoferrin on iron status [46–48].

Support for LfR mediating uptake of iron from lactoferrin was obtained by transfecting human intestinal Caco-2 cells with the LfR gene; transfected cells showed significantly higher uptake of both lactoferrin (labeled with ^{125}I) and iron (labeled with ^{59}Fe) than mock-transfected cells [37]. We have also shown recently that both siRNA silencing of the LfR gene as well as antibody blocking of LfR in Caco-2 cells significantly decrease lactoferrin uptake [Jiang et al., in preparation]. The N1 domain of lactoferrin appears to be critical for internalization and nuclear localization [49], but the segment of the molecule affecting gene transcription has not yet been characterized.

ABSORPTION OF IRON FROM FERRITIN

In an attempt to sustainably increase the intake of dietary iron in large population groups vulnerable to iron deficiency, biofortification of major staple crops, e.g., rice, legumes, and maize, is under development and evaluation [50]. Such biofortification can be achieved by conventional plant breeding methods, selecting for high iron varieties. Legumes such as soybeans, peas, lentils, and beans contain a plant form of ferritin ("phytoferritin") which is similar to ferritin found in iron stores in humans and other animals [51–54]. A considerable proportion of the total iron content of soybeans is in ferritin [55,56], and since such plants can be selected for high ferritin content, there has been interest in evaluating the bioavailability of iron bound to ferritin. This has been further emphasized by the possibility of expressing high levels of ferritin in plants normally low in ferritin (e.g., rice) by genetic engineering [57–59]. Ferritin is a unique protein with regard to its high metal-binding capacity; each protein can bind up to 4500 atoms of iron within its so-called "iron core" [60]. Biofortification with iron using either approach of enhancing the ferritin content of plants would be a sustainable way to increase iron consumption in areas with habitually low intake of dietary iron.

We have shown by radioisotope studies that iron is well absorbed from both animal and plant ferritin, and that it is equal to iron in the form of ferrous sulfate (positive control) [61,62]. Some earlier studies had shown low bioavailablity of iron from ferritin, but this was likely due to methodological problems due to the labeling method used or to ferritin sources not representative of dietary ferritin [63–65]. However, when using a validated extrinsic labeling method and more "native" forms of ferritin, iron was found to be well utilized [61,62].

In order to systematically evaluate dietary factors affecting the absorption of ferritin-bound iron, e.g., phytic acid, tannins, ascorbic acid, and calcium, we used a human intestinal Caco-2 cell model

[66]. We found that these intestinal cells take up iron from ferritin in a receptor-mediated manner; i.e., binding and uptake are saturable and the process is blocked by inhibitors of endocytosis [67]. Another recent study supports this concept [68]. Ferritin receptors have been described in tissues such as liver [69] and placenta [70], and it is not surprising that they are also found in the small intestine. It is therefore possible that dietary iron bound to ferritin is taken up in the small intestine by a receptor-mediated process. This was supported by the fact that inhibitors and enhancers of non-heme iron uptake had no effect on iron uptake from intact ferritin by Caco-cells [66]. It was further supported by our observation that ferritin exposed to digestion in vitro is relatively resistant against proteolytic enzymes [66]. Previous studies have also shown that ferritin is unusually stable to heat, pH, and denaturing agents [71]. Although ferritin ultimately was degraded by a combination of low pH and gastric and pancreatic enzymes [66], and uptake of the released iron now being affected by inhibitors and enhancers of iron absorption, there are may conditions that may limit digestion of ferritin, e.g., achlorohydria, diets with high buffering capacity, and diets in which ferritin is in a less accessible compartment. Whether ferritin is internalized in intact form and the protein subunits and the iron core are processed intracellularly separately or whether they go together to an intracellular compartment is not yet known. Further, the putative ferritin receptor has not yet been cloned or characterized. From an iron absorption perspective, however, it is possible that some ferritin in the diet may remain intact and interact with intestinal ferritin receptors, while some ferritin may be digested and its released iron absorbed via the DMT-1 pathway. The extent to which each pathway is responsible for iron uptake from ferritin is not known, but it is apparent that net iron absorption from ferritin is high [61,62].

In conclusion, iron may be taken up from foods by several different mechanisms (Fig. 1), each being affected by various conditions.

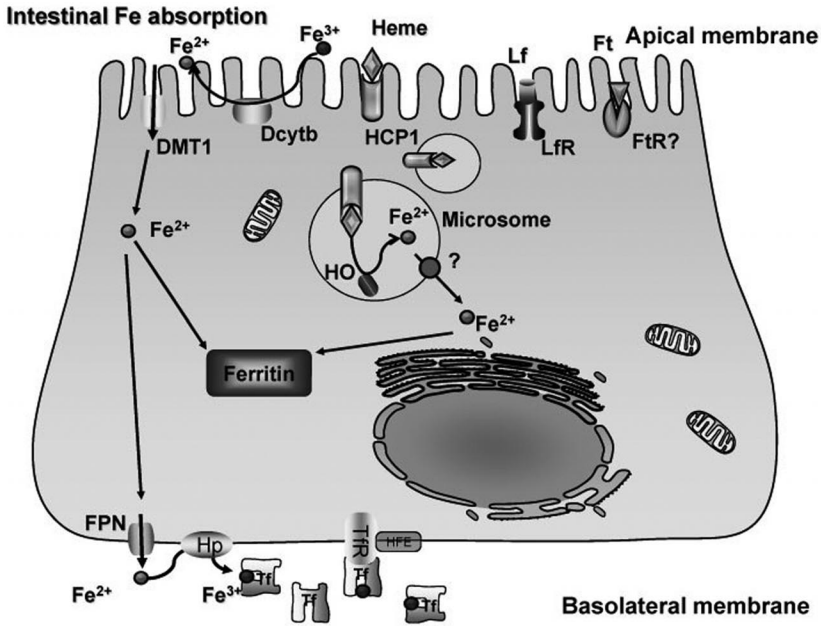

Fig. 1 Different pathways for absorption of iron from foods.

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