Eur J Nutr (2011) 50:637–643
DOI 10.1007/s00394-011-0173-8

ORIGINAL CONTRIBUTION

Effect of dietary protein on heme iron uptake by Caco-2 cells

Pía Villarroel · Sebastián Flores · Fernando Pizarro · Daniel López de Romaña · Miguel Arredondo

Received: 26 June 2010 / Accepted: 26 January 2011 / Published online: 16 February 2011

Abstract
Objective To study heme iron bioavailability and the role of dietary protein (animal and vegetable) on iron uptake using an in vitro model (Caco-2 cell line).
Methods Caco-2 cells were seeded in bicameral chambers with different animal (beef, chicken or fish) or vegetable (peas, lentils, and soybeans) proteins or with pure animal (collagen and casein) or vegetable (gliadin, zein, and glutein) protein extracts. The effect of each protein over heme iron absorption was assessed.
Results Intact heme uptake was higher than either heme plus albumin or digested heme plus albumin, but lower than digested heme. White meal exerted the highest inhibitory effect on hemin uptake. Heme iron uptake decreased in the presence of all legume extracts, but was not significantly different among them (one-way ANOVA, NS). Pure animal (collagen and casein) and vegetable (zein and glutelin) proteins increased heme iron uptake, except for gliadin.
Conclusion Animal and vegetable protein in general decreased heme iron uptake. However, purified animal and vegetable protein induce an increase in heme iron uptake.

Keywords Heme absorption · Bioavailability · Uptake · Transport · Caco-2 cells

P. Villarroel · S. Flores · F. Pizarro · D. L. de Romaña · M. Arredondo (✉)
Micronutrient Laboratory, INTA, University of Chile, El Líbano 5524, 138-11 Macul, Santiago, Chile
e-mail: marredon@inta.cl

Introduction

Iron is essential for the survival of all living organisms. However, iron must be bound to proteins to prevent tissue damage from free radical formation [1]. Total body iron concentration is maintained in part by a controlled metabolic balance of its absorption within the enterocytes [2, 3]. Using intrinsically labeled hemoglobin with ^{59}Fe obtained from rabbits, it has been demonstrated that iron from this source is incorporated into erythrocytes 14 days after ingestion and therefore is absorbed [4] and that absorption occurs mainly in the proximal section of the duodenum [5]. Iron can be the lost into the lumen of the gut when the mucosal cells are sloughed from the villus in a selective manner so that needed iron is conserved and the excess is eliminated [6]. During iron deficiency, the excretory mechanism becomes less active so that the rate for iron accumulation is further increased [7].

Dietary iron is present in food both as inorganic (ferrous and ferric compounds) and as organic forms, mainly in red meat as heme iron [8]. Heme iron is freed from hemoglobin and myoglobin by pancreatic enzymes [9, 10]. Heme is absorbed directly as the intact iron porphyrin complex [2]. The transport process is mediated by a specific protein, HCP1 (Heme Carrier Protein 1), which is located on the apical face of the membrane of the enterocyte [11, 12]. Although heme iron represents a small proportion of total dietary iron, its absorption is much higher (20–30%) and is less affected by other components of the diet. Furthermore, heme iron uptake is not competitive with that of non-heme iron [13]. As for inorganic iron, heme uptake is also favored by the presence of meat in the diet, possibly due to the contribution of certain amino acids and peptides released by digestion, which maintain heme iron soluble, and therefore available for absorption [14]. A certain

proportion of the heme iron in meat is denatured during cooking [15]. Contrary to what has been observed for inorganic iron, ascorbic acid has little effect on the absorption of heme iron, probably due to the reduced availability of coordination links [3]. Calcium reduces the absorption of heme iron, interfering in the transfer of the metal from the mucosal cell, but not in its entry [16].

Hemin, iron (III) protoporphyrin chloride (IX), is produced when heme is released from the globular chain of hemoproteins, such as hemoglobin and myoglobin, and the fifth coordination position of its iron atom is occupied by a chloride or a hydroxyl anion [17]. Contrary to heme, in which iron is in its reduced state (Fe^{+2}), in hemin iron is in its oxidized state (Fe^{+3}) [18]. Nevertheless, the main difference between the two molecules is that heme is bound to proteins. Hemin is extracted from blood, being less than 2% of red blood cells. It has been observed that products of globin degradation are important to maintain heme in a depolymerized form so that heme may be available for absorption [19]. Furthermore, these products facilitate non-heme iron absorption [19]. However, it is not known whether other proteins of the diet produce these same effects. Hemoglobin has been used in iron-fortified food programs, but it has negative effects on the organoleptic characteristics of the product. The mixture of heme with other proteins of animals or vegetables origin might be used to produce products that have a higher acceptability.

The use of cell cultures to study the bioavailability of iron in humans has increased lately [20]. Caco-2 cells are derived from colon cancer cells [21] and are used as a model of the intestinal epithelium [22]. These cells spontaneously differentiate into polarized monolayers with a well-developed apical membrane with microvilli and associated enzymes [23]. The Caco-2 model has been used in a wide range of cellular studies, particularly in the exploration of mechanisms [24, 25] and the regulation of iron absorption [26, 27] and in the assessment of iron bioavailability [28–31]. Uptake of heme iron by Caco-2 cells is a saturable and temperature-dependent process [32], making its use a promising way of measuring the physiological uptake of iron in mucosal cells.

A mix of heme with other proteins, such as those from animals or vegetables, could be used to fortify products which have a higher acceptability than those fortified with hemoglobin alone. Furthermore, given that it is unknown whether other proteins have the same effect as that of globin degradation products on heme and non-heme iron absorption or uptake, the aim of this study was to evaluate the role of dietary protein, both animal and vegetable, over intestinal heme iron uptake, using the Caco-2 cell model.

Materials and methods

Cell culture

Caco-2 cells were obtained from the passage 18 of ATCC collection (American Type Culture Collection, Rockville, Maryland, USA). All experiments were carried out during passages 25–30. The cells were grown in Iscove′s Medium (modified Dulbecco medium without iron, Gibco, BRL) with 10% fetal bovine serum (Sigma, St. Louis, MO) and a 1% antibiotic–antimycotic solution (Gibco, BRL). Cells were maintained at 37 °C and 5% CO_2. When cells reached 80–100% confluence, they were trypsinized and seeded in bottles of 25 cm^2 (Corning Costar Corporation, Cambridge, MA) at a density of 25,000 cells/ml for continued growth. For heme iron uptake experiments, cells were seeded on collagen-treated membranes (polytetrafluoroethylene) in bicameral inserts (transwell, 6 wells, 24 mm in diameter, 0.4-μm pore size, Corning Inc, New York, USA) at a density of 20,000 cells/cm^2. A confluence of 90–100% with tight intercellular junction was assessed 12–14 days after seeding using the method of phenol red [29], and the layers of confluent cells were used for hemin uptake experiments. Also, TEER (transepithelial electric resistance) was measured to check monolayer formation. A bicameral chamber with a TEER over 250 Ω * cm^2 was used.

Isolation of proteins

Prior to in vitro procedures, the protein content of all animal and vegetable proteins and prepared digest was determined by the Kjeldahl method [33]. Total iron content of the different proteins was determined after acid digestion [34] and determined with an atomic absorption spectrophotometer with graphite furnace (Simaa 6100, Perkin Elmer, MA, USA). Because meat contains both non-heme iron and heme iron, heme iron concentration was determined by the difference between total iron and non-heme iron which was measured by Rebouche method [35]. Briefly, each food was digested with trichloroacetic acid and hydrochloric acid. The total iron content corresponding to heme iron in each meal was standardized to 1.1 mg by the addition of hemin (Sigma, St. Louis, MO, USA) dissolved in 0.1 M NaOH (Merck, Darmstadt, Germany). This value corresponds to one serving of a 100 g of steak or each food. No heme Fe was added to the food when the concentration of intrinsic iron was 1.1 mg ($\approx$20 μM).

Collagen, albumin, casein, gliadin, and zein were purchased at their highest commercially available purity (Sigma), whereas glutelin was isolated from rice flour [36]. Lentil and pea creams were prepared according to the method by Bamdad [37] and to the method by Tomoskozi

[38], respectively. The soy isolate was obtained from ALPHA 8® (The Solae Company, St. Louis, MO).

Isotopic labeling of hemin

The iron isotope ^{55}Fe was used as a tracer (6.1 GBk/mg; NEN, Life Science Products, Boston, MA, USA). Labeled hemoglobin was prepared from red cells obtained from two 3-month-old New Zealand rabbits that had received an intravenous injection of 74 MBq of ^{55}Fe (Perkin Elmer, MA, USA) as ferric citrate diluted in saline solution. After a period of 15 days, the animals were anesthetized with intravenously administered sodium thiopental at a dose of 25 mg/kg of weight and subsequently killed by exsanguination. The protocol was approved by the Bioethics Committee of the Institute of Nutrition and Food Technology. Finally, the blood was washed with saline to obtain a concentrate of red blood cells.

Hemin was extracted using a solvent extraction mixture of $SrCl_2$ 2% glacial acetic acid: acetone (1:3 proportion). Briefly, samples were warmed up to the boiling point. The result of this extraction was filtered to remove the precipitate composed of proteins and other cellular debris. The solid remain were washed 2 times with the same extraction solvent, to recover any hemin that might still be present in the protein. The solvent was first heated at 100 °C, cooled and then hemin was precipitated. The salt was recovered by centrifugation at 2,500 rpm for 20 min, dried, and subsequently washed with acetic acid/water (1:1), absolute ethanol and diethylether. A dilution of 0.1 g/L of hemin was prepared to determine iron concentration by atomic absorption spectrophotometry (Perkin Elmer 2280, MAS, USA) and ^{55}Fe specific activity with a liquid scintillation counter (Canberra Packard TRI-CARB Company 1600 TR). Furthermore, absorption spectra were compared with respect to a commercial hemin (Sigma, St. Louis, MO, USA). Maximum absorption was observed at the same wavelengths (360/380 and 400 nm) that agree with results obtained by Lombardo et al. [39]. Hemin used in uptake studies had a final activity of 0.21 μCi of ^{55}Fe/mg and a final iron concentration of 120 μg/mg.

In vitro digestion

Pepsin and pancreatin enzymes and bile salts extract (Sigma, St Louis, MO, USA) were used for in vitro digestion. Each sample was digested in triplicate. Bovine, poultry, and fish samples (150 g) were liquefied with 200 mL of deionized water using a Minipimer/Mixer (MR 4050-HC-V Braun, Darmstadt, Germany). Hemin chloride in 0.1 M NaOH was added to give a total iron of 1.1 mg, and the in vitro digestion was performed. The pH of the mixture was taken to 2 with 5 M HCl, 10 mL of a pepsin solution [0.08 g (2,500 U/mg proteins) per ml of 0.1 M HCl] was added and subsequently incubated for 1 h at 37 °C in a bath with agitation at a speed of 90 rpm to simulate gastric digestion. After incubation, to emulate duodenal digestion pH was raised to 6 by slowly adding 0.1 M $NaHCO_3$, then 50 mL of a pancreatin-bile salt solution was added [0.12 g of extracts of bile salts and 0.02 g of pancreatin (4 times the activity USP) in 5 mL of 0,1 M $NaHCO_3$] and incubated for 30 min at 37 °C. After incubation, the mixture was chilled to stop pancreatic activity. The digests were subjected to centrifugation at 5,000*g* for 30 min.

All proteins were diluted with deionized water (1 g of protein in 10 mL) and subjected to in vitro digestion with pepsin and pancreatin. The digest was diluted to 10% (g protein/g water) before digestion. All supernatants were subjected to protein determination by Lowry method [40]. To study the effect of the protein digests on hemin uptake, we followed the same protocol, and the solutions obtained were added to Caco-2 cells cultures with or without albumin to observe whether they behaved like undigested hemin.

Hemin-Fe uptake studies

The day of the experiment, Caco-2 cells monolayers in bicameral chamber were washed twice with PBS (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 $7H_2O$ 4.3 mM; KH_2PO_4 1.4 mM; pH 7.3), then uptake buffer (50 mM HEPES; 130 mM NaCl; 1 mM $MgSO_4$; 1 mM $CaCl_2$; 10 mM KCl; pH 7.0) was added to each well of in the apical (1.5 ml) and basolateral (2.5 ml) side. Subsequently, hemin-^{55}Fe was added in the apical medium with a protein extract to get a ^{55}Fe concentration of 100 μM (0.28 kBq per insert) and 2 g protein/L. An aliquot (100 μL in triplicate for each sample) was separated to assess basal radioactivity. The samples were incubated for 90 min at 37 °C and 5% CO_2. At the end of the incubation time, an apical and basolateral aliquot were recovered to measure their radioactivity (100 μL in triplicate for each sample). The monolayers were washed twice with 5% albumin in PBS to remove non-specific bound hemin. To recover cell monolayers, the membranes were cut out with a scalpel and immersed in a 1.5-mL tube with 1 mL of scintillation liquid. The radioactivity from ^{55}Fe in the membrane was measured in using a scintillation counter (Packard Canberra Company). In each plate, one insert was used as blank (protein-free hemin), to calibrate inter-plate differences.

Statistical analysis

In all experiments, the variables were tested in triplicate. Statistical analysis of each variable was performed using

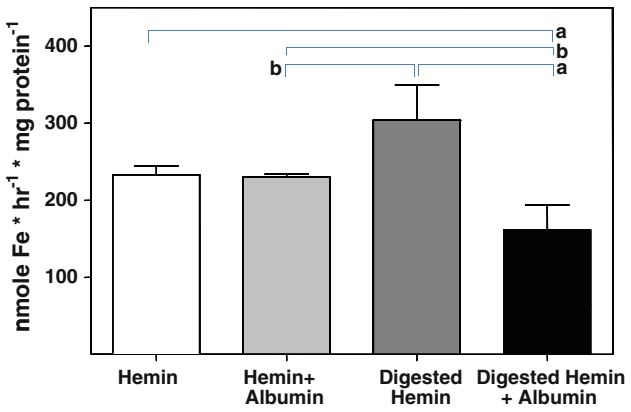

Fig. 1 Hemin and digested heme uptake: effect of albumin. Undigested hemin and digested hemin uptake in Caco-2 cells with and without albumin in the medium. Values shown are mean ± S.E.M ($n = 5$). One-way ANOVA, *a* $p < 0.01$, *b* $p < 0.05$. Heme uptake is expressed as nmole of heme * mg protein^{-1} * h^{-1}

the program Graphpad Prism® v4.03 for Windows (Graphpad Software, CA, USA). One-way ANOVA was used to determine significant differences and Tukey multiple comparison to establish differences between the groups. Results are expressed as average ± standard deviation. The statistical significance was defined as $p < 0.05$.

Results

Effect of hemin digestion and effect of albumin on heme uptake

Heme uptake was measured once and its value was used for all comparisons. Heme iron uptake was not significantly higher than heme plus albumin uptake; however, it was significantly higher than digested heme plus albumin uptake (one-way ANOVA, $p < 0.003$) and was not significantly lower than digested heme uptake (Fig. 1).

Effect of animal protein as beef, chicken or fish on heme uptake

Heme iron uptake decreased according to animal protein discoloration (one-way ANOVA, $p < 0.001$ for all differences) (Fig. 2a).

Effect of protein derived from legumes as pea, lentil, or soybean in the uptake of heme

Heme Fe uptake also decreased in the presence of all legumes proteins extracts (one-way ANOVA, $p < 0.001$ for all differences). (Fig. 2b).

Effect of purified animal or vegetable protein on heme uptake

The presence of pure animal protein as either casein or collagen produced a significant increase in heme Fe uptake (one-way ANOVA, $p < 0.004$) (Fig. 3a). Purified zein produced an increase in heme uptake, and glutelin has no significant effect, while gliadin significantly decreased heme uptake (one-way ANOVA, $p < 0.001$) (Fig. 3b).

Discussion

Heme iron uptake depends on intraluminal factors that modulate the internalization of the molecule from the protoporphyrin ring. The effect of the different conditions and interactions is still in discussion. In the present study, the digestion of hemin with pepsin and pancreatin had no effect on the uptake of heme iron; however, albumin significantly decreased the uptake of digested hemin and reduced somewhat, if not significantly, the uptake of undigested hemin. The mechanism by which albumin inhibits the uptake of heme iron is unknown.

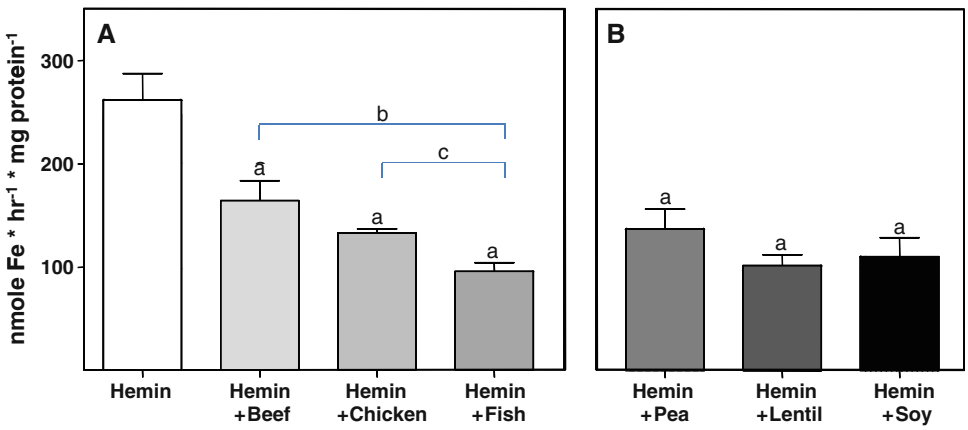

Fig. 2 Heme Fe uptake from animal and vegetable protein extract. Heme uptake was measured in the presence of animal protein extracts (beef, chicken and fish) or vegetable protein extracts (pea, lentil and soybeans). Values shown are mean ± S.E.M ($n = 5$). One-way ANOVA, *a* $p < 0.001$, respect to heme, *b* $p < 0.001$ and *c* 0.01. Heme uptake is expressed as nmole of heme * mg protein^{-1} * h^{-1}

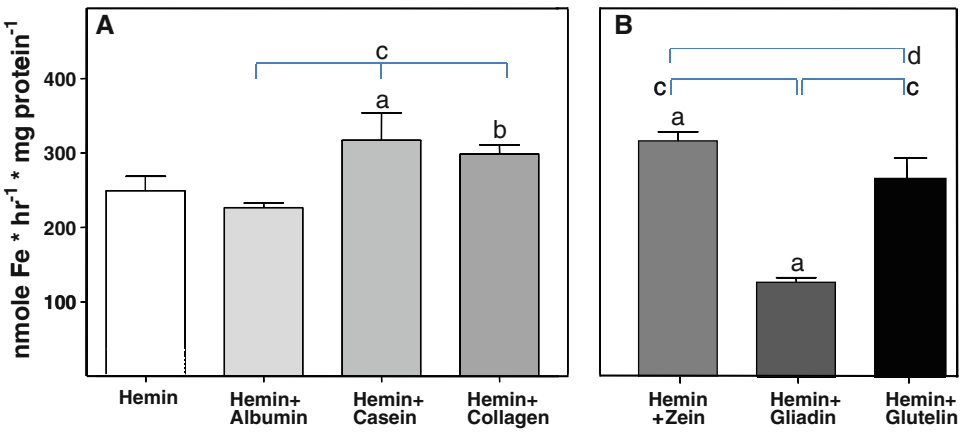

Fig. 3 Heme uptake in the presence of purified animal or vegetable protein. Heme uptake was measured in the presence of purified animal protein (albumin, casein, and collagen) or purified vegetable protein (zein, gliadin and glutelin). Values shown are mean ± S.E.M ($n = 5$). One-way ANOVA, **a** $p < 0.01$ and **b** $p < 0.05$, respect to heme; **c** $p < 0.001$ and **d** $p < 0.05$. Heme uptake is expressed as nmole of heme * mg protein^{-1} * h^{-1}

It has been shown that iron absorption from vegetable and animal foodstuffs, where radioactive iron was incorporated biologically into a single food, ranged from 3 to 8% and 8 to 16%, respectively [41]. In a study where iron absorption was measured from vegetable and animal proteins, absorption values from wheat, corn, black beans, lettuce, and spinach were lower than values from soybeans, fish, veal, and hemoglobin [42]. In the present study, hemin uptake decreased in the presence of beef, chicken and fish, which indicates that meat inhibits luminal hemin iron uptake. Also, in the present study, the meat/protein ratio was directly proportional to the presence of myoglobin, and white meat inhibited hemin uptake more strongly than red meat. Thes results differ with those of Hallberg et al. [43], who conclude that meat enhances the absorption of heme iron. The authors hypothesized that meat has an influence on both non-heme and heme absorption via the stimulation of food digestion, leading to a more efficient release of either form, which therefore results in an increased availability for absorption. Thus, meat counteracts luminal factors that inhibit non-heme iron absorption and enhances heme iron absorption [43]. The likely mechanism for the aforementioned effect is the formation of a luminal carrier that transports the iron to the mucosal cell membrane [44].

With respect to legume proteins, the results of the present study are categorical; all of them exerted a significant negative effect on the absorption of hemin iron. This is contrary to the results by Lynch et al. [45], where partial substitution of beef with soy flour improved heme iron absorption significantly (27–59%). This study provides new evidences and confirms the inhibitory effect of soy on heme iron absorption and bioavailability. These results are relevant because of the increasing consumption of soy, specially in developed countries.

Purified animal proteins favored the uptake of hemin, suggesting that certain proteins and/or peptides are necessary to activate the uptake mechanisms in the enterocyte [14]. Nevertheless, casein in milk has been shown to reduce heme iron bioavailability [16].

Gliadin, the vegetable protein from rice, inhibited the uptake of hemin, whereas zein favored its uptake. Therefore, it appears that the effect of purified vegetable proteins on the bioavailability of heme iron is dependent on the plant origin.

Heme iron solubility significantly increases with the presence of proteins and, in general, iron-rich foods contain high amounts of proteins, which suggests that an increase in hemoglobin hydrolysis might lead to an increase in iron uptake [46]. This might explain the different effects obtained from hemin in the present study. It would seem that the heme iron matrix is important to favor heme iron uptake. The differences in the uptake of heme and hemin could be explained by the presence of molecules that modulate their bioavailability. Although, hemin and heme use the same carrier for their uptake [47], during digestion, heme uptake is probably facilitated by proteins released from meat and hemoglobin. These proteins likely prevent heme polymerization [48, 49]. The digestion products are probably able to bind "free" inorganic iron from meat itself or from other meals [50]. Also, when myoglobin from meat is digested, low molecular weight non-heme iron compounds could be the major degradation products [51]. Given that hemin does not have accompanying proteins, it might differ from heme iron in its mode of uptake.

In conclusion, animal and vegetable protein in general decreased hemin iron uptake. However, purified animal protein and vegetable protein induce an increase in hemin iron uptake. As hemin is a chloride of heme, the formation of chelates and the affinity of binding to different proteins might influence its uptake by Caco-2 cells. Hemin interactions with different proteins under in vivo conditions could be much more complex and complicated.

Acknowledgments The authors are grateful for support from Fondo Nacional de Desarrollo Científico y Tecnológico de Chile (FONDECYT), grant number 1095038.

References

1. McCord JM (1998). Iron, free radicals, and oxidative injury. Semin Hematol 35: 5–12. Review
2. Turnbull A, Cleton F, Finch CA (1962) Iron absorption. IV. The absorption of hemoglobin iron. J Clin Invest 41:1897–1907
3. Hallberg L, Solvell L (1967) Absorption of hemoglobin iron in man. Acta Med Scand 181:335–354
4. Callender ST, Mallett BJ, Smith MD (1957) Absorption of haemoglobin iron. Br J Haematol 3:186–192
5. Conrad ME, Umbreit JN, Moore EG (1999) Iron absorption and transport. Am J Med Sci 318:213–229
6. Conrad ME, Weintraub LR, Crosby WH (1964) The role of the intestine in iron kinetics. J Clin Invest 43:963–974
7. Crosby WH, Conrad ME Jr, Wheby MS (1963) The rate of iron accumulation in iron storage disease. Blood 22:429–440
8. Carpenter CE, Mahoney AW (1992) Contributions of heme and nonheme iron to human nutrition. Crit Rev Food Sci Nutr 31:333–367
9. Conrad ME, Benjamin BI, Williams HL, Foy AL (1967) Human absorption of hemoglobin-iron. Gastroenterology 53:5–10
10. Monsen ER, Hallberg L, Layrisse M, Hegsted DM, Cook JD, Mertz W, Finch CA (1978) Estimation of available dietary iron. Am J Clin Nutr 31:134–141
11. Worthington MT, Cohn SM, Miller SK, Qi Luo RQ, Berg CL (2001) Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. Am J Physiol Gastrointest Liver Physiol 280:G1172–G1177
12. Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, Halliday N, Khan Y, Warley A, McCann FE, Hider RC, Frazer DM, Anderson GJ, Vulpe CD, Simpson RJ, McKie AT (2005) Identification of an intestinal heme transporter. Cell 122:789–801
13. Bjorn-Rasmussen E, Hallberg L, Isaksson B, Arvidsson B (1974) Food iron absorption in man. Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from the whole diet. J Clin Invest 53:247–255
14. Uzel C, Conrad ME (1998) Absorption of heme iron. Semin Hematol 35:27–34
15. Schricker BR, Miller DD (1983) Effects of cooking and chemical treatment on heme and non-heme iron in meat. J Food Sci 48:1340–1344
16. Hallberg L, Rossander-Hulthen L, Brune M, Gleerup A (1993) Inhibition of haem-iron absorption in man by calcium. Br J Nutr 69:533–540
17. Tenhunen R, Tokola O, Linden IB (1987) Haem arginate: a new stable haem compound. J Pharm Pharmacol 39:780–786
18. Lee KS, Raymond LD, Schoen B, Raymond GJ, Kett L, Moore RA, Johnson LM, Taubner L, Speare JO, Onwubiko HA, Baron GS, Caughey WS, Caughey B (2007) Hemin interactions and alterations of the subcellular localization of prion protein. J Biol Chem 282:36525–36533
19. Conrad ME, Umbreit JN (2000) Iron absorption and transport-an update. Am J Hematol 64:287–298
20. Au AP, Reddy MB (2000) Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. J Nutr 130:1329–1334
21. Fogh J, Wright WC, Loveless JD (1977) Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst 58:209–214
22. Artursson P (1990) Epithelial transport of drugs in cell culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J Pharm Sci 79:476–482
23. Alvarez-Hernandez X, Nichols GM, Glass J (1991) Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. J Biochim Biophys Acta 1070:205–208
24. Han O, Failla M, Hill A, Morris E, Smith J (1995) Reduction of Fe(III) is required for uptake of nonheme iron by Caco-2 cells. J Nutr 125:1291–1299
25. Nuñez MT, Alvarez X, Smith M, Tapia V, Glass J (1994) Role of redox systems on Fe3 + uptake by transformed human intestinal epithelial (Caco-2) cells. Am J Physiol 267:C1582–C1588
26. Alvarez-Hernandez X, Smith M, Glass J (1994) Regulation of iron uptake and transport by transferrin in Caco-2 cells, an intestinal cell line. Biochim Biophys Acta 1192:215–222
27. Gangloff M, Lai C, Van Campen DR, Miller DD, Glahn RP (1996) Ferrous iron uptake but not transfer is down-regulated in Caco-2 cells grown in high iron serum-free medium. J Nutr 126:3118–3127
28. Nunez MT, Tapia V (1999) Transferrin stimulates iron absorption, exocytosis, and secretion in cultured intestinal cells. Am J Physiol 276:C1085–C1090
29. Garcia MN, Flowers C, Cook JD (1996) The Caco-2 cell culture system can be used as a model to study food iron availability. J Nutr 126:251–258
30. Glahn RP, Lai C, Hsu J, Thompson JF, Guo M, Van Campen DR (1998) Decreased citrate improves iron availability from infant formula: application of an in vitro digestion/Caco-2 cell culture model. J Nutr 128:257–264
31. Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD (1998) Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. J Nutr 128:1555–1561
32. Arredondo M, Kloosterman J, Núñez S, Segovia F, Candia V, Flores S, Le Blanc S, Olivares M, Pizarro F (2008) Heme iron uptake by Caco-2 cells is a saturable, temperature sensitive and modulated by extracellular pH and potassium. Biol Trace Elem Res 125:109–119
33. Chaney AL, Marbach EP (1962) Modified reagents for determination of urea and ammonia. Clin Chem 8:130–132
34. Olivares M, Pizarro F, de Pablo S, Araya M, Uauy R (2004) Iron, zinc, and copper: contents in common Chilean foods and daily intakes in Santiago, Chile. Nutrition 20:205–212
35. Rebouche CJ, Wilcox CL, Widness JA (2004) Microanalysis of non-heme iron in animal tissues. J Biochem Biophys Methods 58:239–251
36. Udaka J, Koga T, Tsuji H, Kimoto M, Takumi K (2000) Efficient extraction and some properties of storage proteins (prolamin and glutelin) in ancient rice cultivars. J Nutr Sci Vitaminol (Tokyo) 46:84–90
37. Bamdad F, Hossein Goli A, Kadivar M (2006) Preparation and characterization of proteinous film from lentil (*Lens culinaris*): Edible film from lentil (*Lens culinaris*) Food. Res. Internat 39:106–111
38. Tomoskozi S, Lasztity R, Haraszi R, Baticz O (2001) Isolation and study of the functional properties of pea proteins. Nahrung 45:399–401
39. Lombardo ME, Lombardo ME, Araujo LS, Ciccarelli AB, Battle A (2005) A spectrophotometric method for estimating hemin in biological systems. Anal Biochem 341:199–203
40. Lowry OH, Rosebrough N, Farr A, Randall R (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
41. Layrisse M, Martinez-Torres C, Roche M (1968) Effect of interaction of various foods on iron absorption. Am J Clin Nutr 21:1175–1183
42. Layrisse M, Cook JD, Martinez-Torres C, Roche M, Kuhn IN, Finch CA (1969) Food iron absorption: a comparison of vegetable and animal foods. Blood 33:430–443
43. Hallberg L, Bjom-Rasmussen E, Howard L, Rossander L (1979) Dietary heme iron absorption. A discussion of possible

mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. Scand J Gastroenterol 14:769–779
44. Bjorn-Rasmussen E, Hallberg L (1979) Effect of animal proteins on the absorption of food iron in man. Nutr Metab 23:192–202
45. Lynch SR, Dassenko SA, Morck TA, Beard JL, Cook JD (1985) Soy protein products and heme iron absorption in humans. Am J Clin Nutr 41:13–20
46. Vaghefi N, Nedjaoum F, Guillochon D, Bureau F, Arhan P, Bouglé D (2002) Influence of the extent of hemoglobin hydrolysis on the digestive absorption of heme iron. An in vitro study. J Agric Food Chem 50:4969–4973
47. Dang TN, Bishop GM, Dringen R, Robinson SR (2010) The putative heme transporter HCP1 is expressed in cultured astrocytes and contributes to the uptake of hemin. Glia 58:55–65
48. Layrisse M, Martinez-Torres C (1972) Model for measuring dietary absorption of heme iron: test with a complete meal. Am J Clin Nutr 25:401–411
49. Conrad ME, Weintraub LR, Sears DA, Crosby WH (1966) Absorption of hemoglobin iron. Am J Physiol 211:1123–1130
50. Hazell T, Ledward DA, Neale RJ (1978) Iron availability from meat. Br J Nutr 39:631–638
51. Hazell T, Ledward DA, Neale RJ (1982) Hemoglobin and meat iron absorption. Am J Clin Nutr 36:187–189