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The effect of cysteine and 2,4-dinitrophenol on heme and nonheme absorption in a rat intestinal model

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Previous studies have showed that purified heme iron forms insoluble polymers that are poorly absorbed. The presence of peptides and of amino acids maintaining heme iron in a soluble form could improve its bioavailability. The digestive uptake and transfer of a concentrated hydrolysate of heme peptides (HPH) and of iron gluconate (Gluc) at 100 μM were compared in vitro in a Ussing chamber. The effects of an enhancing amino acid (L-cysteine) on the uptake and transfer of both forms were assessed. An inhibitor of the oxidative phosphorylation (2,4-dinitrophenol; DNP) was used to differentiate the active and passive mechanisms of the absorption. The mucosal uptake (%Tot) and enterocyte transfer (%S) of the two sources of iron did not differ. DNP significantly reduced %Tot and %S of both forms. Cysteine significantly enhanced %Tot and %S of HPH and Gluc, partly corrected the inhibition exerted by DNP on %Tot of HPH and %S of both forms, and fully restored %Tot of Gluc. In presence of peptides produced by globin hydrolysis, the absorption of hemoglobin iron was efficient; it was mostly energy dependent and, therefore, should have occurred by a regulated transcellular pathway. Cysteine enhanced the passive uptake of iron and the passive processes involved in the enterocyte transfer of the common pool made of both sources (heme and nonheme) of iron. These results showed that heme iron can be purified and concentrated without impairing its digestive absorption, provided it remains in presence of peptides or amino acids. (J. Nutr. Biochem. 11:562–567, 2000)

Keywords: heme; iron; absorption; mechanisms; cysteine; dinitrophenol

Introduction

The digestive comfort of heme iron is good;[1] it does not interfere with trace elements such as zinc.[2,3] Its high bioavailability depends on iron stores[2,4–13] and does not depend on dietary factors, with the exception of meat[5,7,10] and, to a lesser extent, calcium.[2,8] Hemoglobin iron has already been used to fortify foods for children.[4,14–16]

Heme iron is absorbed as an intact metalloporphyrin across the intestinal brush border after it has been cleaved from globin by digestive enzymes[17] and is released inside the enterocyte by a heme oxygenase. It then enters a common pool with inorganic iron and enters the bloodstream.[5,6,18]

The iron content of hemoglobin is low (0.35%); concentrating heme by hydrolyzing globin and discarding low molecular weight peptides yields free heme, which forms polymers of large size and poor solubility in the digestive lumen.[6,19] The absorption of heme iron requires the presence of peptides or amino acids from degradation of globin or other dietary proteins that could prevent aggregate formation and increase its absorption.[5–7,19] Cysteine is one of the most efficient amino acids in enhancing the absorption of heme or nonheme iron.[7,20–23] Several reports studied the mechanisms involved in the effects of this amino acid,[24–26] but the precise site of its action remains unknown.

The aim of the present study was to assess the efficiency and the mechanisms of absorption of a concentrated form of heme iron in the presence of peptides issued from the

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Received February 28, 2000; accepted August 14, 2000.

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0955-2863/00/$–see front matter
PII S0955-2863(00)00120-0

enzyme hydrolysis of hemoglobin; this hydrolysate of heme peptides (HPH) has a low peroxidation potential and is stable in response to pH variations in vitro.[27] To precise the mechanisms involved in heme iron absorption, 2,4-dinitrophenol (DNP), an inhibitor of oxidative phosphorylation, was used to assess the active uptake and transfer of iron. The experimental model allowed us to differentiate the specific effects of cysteine on the two steps of iron absorption, namely, uptake and enterocyte transfer.[27–29] Iron gluconate (Gluc) was used as control.

The model we used was the Ussing chamber, which uses live fully organized digestive membranes including the mucus layer that affects the diffusion of iron from the lumen to the enterocyte.[30,31]

Contrary to nonheme iron, the absorption rate of heme iron is similar in rats and in humans; the mechanisms of heme iron absorption and their regulation, depending on the dose and on iron status, are known to occur in the rodent mucosa such as in humans. In both, upregulation of heme iron by iron deficiency is less efficient than of nonheme iron.[12,18,32–35] In rats, iron absorption is less sensitive to dietary factors that are known to affect it in humans;[36,37] therefore, direct human applications cannot be drawn from rat studies, but this model can be used to bring out some expectable trends in human responses.

Materials and methods

Preparation of HPH

Hydrolysates were prepared at the Laboratoire de Technologie des Substances Naturelles (Lille University, Lille, France) as previously described.[27] Bovine hemoglobin was prepared from centrifuged red cells provided by Veos Novo, (Zwevezele, Belgium). Hemoglobin solution obtained by red blood cell hemolysis was adjusted to 5% (w/v) with 10 mM dm^{-3} HCl. Hemoglobin concentration was determined according to the cyanmethemoglobin method.[38] pH was then adjusted to the appropriate hydrolysis pH with 1 M HCl or 1 M NaOH.

Pepsin hydrolysis was carried out at pH 3 at 40°C by addition of porcine pepsin (pepsin A, EC. 3.4.23.1, from porcine stomach mucosa, Sigma Chemical Co., St. Louis, MO USA) with an enzyme/protein ratio of 6% (w/w). pH was maintained constant by using a pH-state (Titroline alpha, Schott Geräte, Hofheim, Germany). The reaction was stopped by adjusting the solution to pH 8 with 1 M NaOH.

Heme was concentrated by membrane ultrafiltration of the hydrolysate; the resulting concentrate of heme and peptides was spray-dried to produce a water-soluble powder containing 96% dry matter (95% protein; 0.50% iron).

Animals

Female Sprague-Dawley rats, 200 to 250 g in weight, were obtained from the Caen University farm. They were housed at 20–22°C in a room with controlled lighting. The rats had free access to a maintenance diet for adult animals (UAR; Villemoisson-sur-Orge, France; Fe = 240 mg/kg diet) and deionized water. Hemoglobin (Hb) values of rats (14.2 ± 0.4 g/dL) were determined by bleeding 1 mL from the retroocular capillary before the experiment.

Eight groups (6 animals/group) were studied. The absorption of heme iron from HPH was compared to nonheme iron provided as Gluc. For each source of iron, the influence of the enhancing amino acid of iron absorption (L-cysteine) and/or of the inhibitor of phosphorylative oxidation, DNP, was tested.

Apparatus

The experiment used a diffusion cell derived from the Ussing chamber model,[28,29] which is made up of two acrylic half-cells separated by the digestive membrane (1.2 cm^2 area); each half-cell contains 3 mL (Marty Technologies; Marcilly-sur-Eure, France). Before the experiments, cells were washed with dilute (1/1000) nitric acid (Merk Laboratories; Nogent-sur-Marne, France) to prevent contamination. The diffusion cells and the media were preheated to 37°C by a block heater that maintained this temperature throughout the experiment.

Perfusion solutes

Ringer Lavoisier solution (Laboratoires Chaix et Marais, Paris, France) was used for luminal and serosal solutions; it is made of Na^+: 139 mEq/L, K^+: 2.7 mEq/L, Ca^{++}: 1.8 mEq/L, HCO^{3-}: 2.4 mEq/L, and Cl^-: 141.4 mEq/L, at a pH of 6.55. Luminal medium was prepared by addition of 100 μM iron as Gluc or HPH. Gluc ($C_{12}H_{22}FeO_{14}$, 2 H_2O) was purchased from Merk Laboratories (Nogent-sur-Marne, France). L-Cysteine (Sigma, Saint Quentin-Fallavier, France) was added as required at previously used concentrations: cysteine/iron (w/w) = 105 (molar ratio 39.3).[39] D-glucose (20 mM) was added to the serosal medium to help maintain tissue viability; mannitol (20 mM) was added to the luminal solution to keep an equal osmolarity of 300 mOsM on each side of the mucosa.[28,29] pH was adjusted to 7–7.5 by addition of chlorhydric acid; Gluc was chosen because its stability at alkaline pH; pH below 6 leads to an aggregation of proteins and peptides.[40]

The absence of Gluc oxidation in luminal compartment during the experiment was controlled by addition of sodium thiocyanate (Sigma, Saint Quentin-Fallavier, France) to a sample of the solute drawn at its beginning and at its end. The reaction yields red Fe^{3+}-cyanate complex whose concentration is directly measured by spectrophotometry (UVIKON 930, Kontron Instruments). When necessary, DNP (Sigma, Saint Quentin-Fallavier, France) was added at a concentration of 500 μmol/L.

Experimental protocol

Animals were unfed overnight before the experiment. They were killed by an intracardiac injection of pentobarbital (Doléthal, Vétoquinol™, Lyon, France). A midline incision was made to expose the intestine. A 2-cm segment of duodenum, immediately distal to the pylorus, was removed and washed in oxygenated Ringer Lavoisier solution. Muscularis mucosa was carefully scraped from the mucosa; the first proximal centimeter of duodenum was opened along its mesenteric border to expose its epithelial surface and was placed on the pins of a half-cell. The matching half-cell was joined to seal the diffusion apparatus.[28,29] Solutes were circulated by a gas lift controlled by valves (O_2 95%/CO_2 5%). A small amount of an antifoaming agent (Silicone 414, Rhodorsil, France) was added to each medium to prevent the development of foam due to gas circulation.

Integrity and viability of the intestinal segment were checked by determination of the transepithelial electrical resistance (TEER) across the membrane (Millicell-ERS, Millipore) throughout the experiment and by histology at its end. TEER was expressed in ohms.cm^2.[41] The experiment lasted 2 hr.

Sample analysis

Luminal and serosal media were analyzed before and at the end of the experiment. The two half-cells were rinsed with diluted nitric

Table 1 Effect of cysteine (Cys) and 2,4-dinitrophenol (DNP) on iron uptake (%Tot) and transfer (%S) by rat intestinal mucosa

Group	%S	%Tot	TEER
Gluc	2.41 ± 0.5	5.20 ± 0.5	27.5 ± 8.4
Gluc + Cys	3.88 ± 0.6[a,b,*]	8.18 ± 1.9[a,b,*]	36.0 ± 4.6
Gluc + DNP	0.77 ± 0.3[a]	2.62 ± 0.5[a]	37.6 ± 15.9
Gluc + Cys + DNP	1.70 ± 0.2[a,c,†]	4.83 ± 0.2[c,†]	34.6 ± 2.8
HPH	1.94 ± 0.3[c]	4.85 ± 1.6[c]	25.0 ± 3.5
HPH + Cys	2.85 ± 0.2[c,d,*]	6.30 ± 1.4[c,d,*]	28.0 ± 5.1
HPH + DNP	0.37 ± 0.1[a,c,d,e]	0.98 ± 0.03[a,c,d,e]	36.1 ± 7.2
HPH + Cys + DNP	0.77 ± 0.3[a,d,e,†]	1.93 ± 0.4[a,*d,e,†]	31.7 ± 3.3

Values are means ± SD (n = 6/group).
For each form of iron (Gluc and HPH), analysis of variance (ANOVA) tests were performed, followed by post hoc Student's t-tests. Corresponding Gluc and HPH groups were compared by Student's t-test: Gluc groups: ANOVA, $P < 0.0001$: [a]different from Gluc ($P < 0.0003$); [b]different from Gluc + DNP and Gluc + Cys + DNP ($P < 0.008$); [c]different from Gluc + DNP ($P < 0.02$). HPH groups: ANOVA, $P < 0.0001$: [d]different from HPH ($P < 0.0002$); [e]different from HPH + Cys ($P < 0.0001$). HPH vs. Gluc: groups sharing the same superscript, $^{*}P < 0.008$; $^{\dagger}P < 0.03$.
No significant difference was observed for TEER.
HPH–heme peptides hydrolysate. Gluc–iron gluconate. TEER–transepithelial electrical resistance of intestinal segments (ohms.cm^2).

acid until no iron could be desorbed from cell walls or digestive membrane.

Mucosa were dried in an oven until their weight was stable, and were digested by incubating with 1 mL nitric acid (65%) for 24 hr. A piece of duodenum immediately distal to the segment studied was used to measure the basal content of iron of digestive membrane.

Iron concentration was measured in luminal and serosal media and in mucosa by atomic absorption spectrometry (Perkin Elmer 1100B, 91400 Villebon ^{S}/ Yvette, France).

After correction for the calculated basal mucosal content was made, the following were calculated: %Tot—the total amount of iron removed from the luminal medium (vs. initial iron quantity) during the experiment, and %S—the total amount transferred across mucosa to the serosal medium (vs. initial iron quantity) during the experiment.

Statistical analysis

Results are expressed as means ± 1 SD. Student's t-test was used to compare %Tot and %S between HPH and Gluc. For each source of iron, the effects of cysteine and DNP on every step of absorption were compared by analysis of variance, followed by Student's t-tests. The significance level was set at $P = 0.05$.

Results

Table 1 and *Figure 1* (results in *Figure 1* are expressed as a percentage of control groups) show the effects of cysteine and/or DNP on iron uptake and transfer by rat mucosa, and on the TEER.

No ferric iron was detected in the luminal compartment of the device during the experiments with Gluc (whether cysteine was present or not), confirming the stability of Gluc at alkaline pH. No significant difference was observed for TEER between groups.

Effect of cysteine

An enhancing effect of cysteine was observed for both Gluc and HPH iron uptake and transfer. Total iron uptake with cysteine was 130% for HPH and 157% for Gluc versus controls. Iron transfer was 147% for HPH and 161% for Gluc versus controls. The addition of cysteine increased the passive part of the metabolic pathway involved in iron absorption. As a result (*Figure 2*), 20.3% of HPH iron uptake and 50.4% of Gluc uptake used passive energy-independent pathways; addition of cysteine to the medium

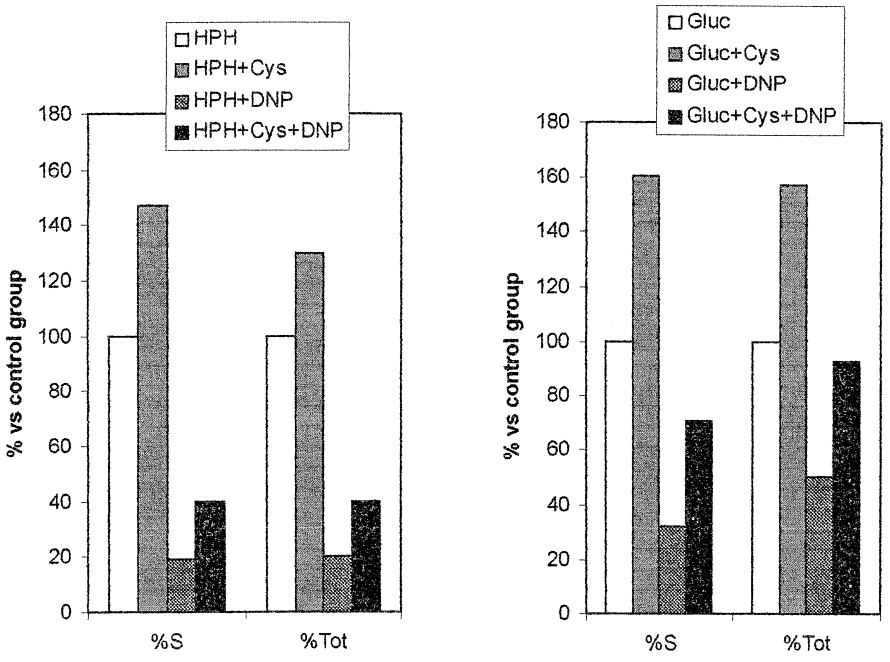

Figure 1 Effect of cysteine (Cys) and/or 2,4-dinitrophenol (DNP) on iron uptake (%Tot) and serosal transfer (%S) by rat duodenum. Values of control groups given in *Table 1* (HPH, heme peptides hydrolysate; Gluc, iron gluconate) are considered as 100%. Significant differences between groups are the same as in *Table 1*.

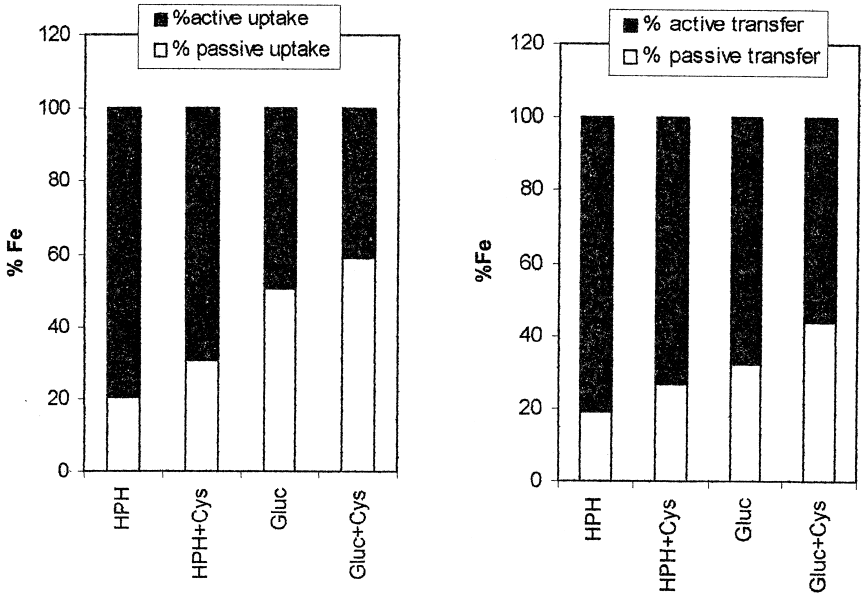

Figure 2 Passive and active metabolic pathways of heme and nonheme iron absorption. 2,4-Dinitrophenol was used as an inhibitor of oxidative phosphorylation to inhibit energy-dependent absorption pathway. Passive absorption (%) is the ratio of residual absorption on 2,4-dinitrophenol to basal absorption. HPH, heme peptides hydrolysate; Gluc, iron gluconate; Cys, cysteine.

increased these levels to 28.3% and 59.1%, respectively. This was also observed in iron transfer to the serosal compartment: 19.1% of HPH iron transfer and 32.0% of Gluc transfer occurred by passive process; cysteine increased these values to 26.9% and 43.8%, respectively.

Effect of DNP

DNP displayed an inhibitory effect on both Gluc and HPH iron absorption. 80% of HPH uptake and 50% of Gluc uptake were inhibited by DNP addition to the luminal medium. Iron transfer was also inhibited by DNP at 80% and 68% for HPH and Gluc, respectively.

Effect of cysteine and DNP

The simultaneous addition of cysteine and DNP to the luminal compartment corrected part of the DNP inhibition of iron uptake and transfer. An increase of 20% for HPH and 40% for Gluc iron uptake and transfer was observed versus groups with DNP alone.

Discussion

The high prevalence of iron deficiency and the low absorption rate of inorganic forms have led to the search for efficient protected forms of iron. Hemoglobin iron is known to be naturally protected from interactions with other nutrients and digestive secretions.[6,42] Attempts to purify heme and increase its concentration usually decrease its digestive solubility and its absorption rate,[19] which seems to depend on the presence of peptides and amino acids produced by globin hydrolysis.[5,7,19] The present study assessed the absorption rate and the different phases of absorption of heme iron, supplied as a bovine hemoglobin HPH. Contrary to heme monomers, this heme-peptide complex remains soluble over a wide range of pH.[27]

HPH iron and Gluc displayed the same basal absorption rate. Cysteine influenced the two steps of iron absorption; as a consequence, it enhanced the absorption of both forms of iron. The inhibition of oxidative phosphorylation by DNP inhibited the uptake and the enterocyte transfer of HPH iron and of Gluc. Addition of cysteine to DNP allowed defining the mode of action of cysteine: It restored the Gluc uptake above its initial level, significantly increased HPH uptake, and displayed a less intense enhancing effect on iron transfer out of the digestive membrane.

The enterocyte uptake of heme iron occurs by a pathway different from inorganic iron: Heme binds a specific receptor and is internalized by endocytosis as a heme-receptor complex.[12,17] The present study showed that this uptake is mostly (80%) energy dependent: Passive absorption of heme can occur by permeation of paracellular tight junctions;[43] some hydroxyl ions can also enter into the heme groups and produce ferric hydroxydes, which leave the porphyrin rings and are passively absorbed.[44]

The lower enhancing effect of cysteine on HPH iron uptake compared to Gluc suggests that HPH is less involved in digestive interactions than Gluc, and confirms the in vitro stability of the HPH;[27] this supports the role of accompanying peptides on the absorption of heme iron because extensive purification lowers its absorption rate.[19] On the other hand, the uptake of inorganic iron occurs by two different processes that are operating simultaneously. The first one involves energy-independent simple or facilitated diffusion; it depends on the free metal concentration on both sides of the epithelial membrane and on the relative solubility of the metal ion in the mucus layer and in the lipid bilayer. It can be a transmembrane movement or take place through a paracellular pathway. The second process is specific and displays saturation kinetics;[5,25,26,45] a specific transporter for divalent cations was characterized and cloned.[46] Even if iron binding to the membrane is passive, its transfer to the cytosol involves its oxidation into ferric iron and uses shuttle proteins such as mobilferrins and integrins.[40] The present study showed that for a luminal iron concentration of 100μM in nondepleted animals, the energy-dependent mechanisms represent 50% of the total uptake of inorganic iron. Previous studies using a metabolic inhib-

itor gave a wide range of results, varying from no inhibition at all[47] to 18.9%[44,45] or 50–60%;[48] a 14.4% inhibition of iron absorption by the colon was also reported.[49] Several assumptions can be made to explain these discrepancies. A lower iron status could have increased the rate of passive absorption;[5] the short time allowed for the experiment in some of these studies could have lessened the energy required to restore ATP consumed during iron transport and, therefore, the effect of DNP.[47] A similar level of active transport (50–60%) was displayed by the perfused duodenal loop model when the study lasted a longer time (2 hr).[48]

Previous studies indicate that cysteine and sulfhydryl compounds enhance iron uptake primarily by reduction and not by increasing solubility;[22–24,50,51] however, in the present study, iron remained in a reduced form in the medium even without addition of cysteine. The polar side chain of the amino acid has also the potential to bind to metal ions by tridentate coordination;[25] cysteine could also influence iron binding to mucins, which are large glycosylated proteins rich in threonine, serine, and cysteine.[30] Cysteine facilitates the cross-linking of mucin molecules[52] that have large capacities for extracting metal ions from the intestinal lumen;[31] binding iron to mucin keeps it soluble and favors its absorption.[30] The presence of cysteine, therefore, could modify the ability of iron to permeate the mucus layer and reach the mucosa. Lastly, iron could be transferred across the membrane as a cysteine-iron complex using the facilitated transport mechanism of amino acids. The active transfer of amino acids into the intercellular spaces creates an osmotic force for fluid flow, triggering enhanced paracellular permeability; however, this study did not display any change in TEER, which could suggest an increase of membrane permeability.

Adding DNP to cysteine gave some information on the mechanisms of action of this amino acid on iron absorption: Cysteine restored the initial uptake of inorganic iron, which is in agreement with the involvement of this amino acid in the different passive luminal and membrane processes reported above.

The Ussing chamber allowed differentiating the effects of DNP and cysteine on the two steps of absorption. Use of DNP significantly lessened the transfer of iron to the serosal side of the digestive membrane; the remaining fraction, which is similar for both forms, could have used the paracellular pathway.[26] Cysteine enhanced iron transfer to serosal medium of two forms. This is explained by the presence of an intracellular pool of iron, common to all absorbed iron forms, after uptaken heme was cleaved by a heme oxygenase.[12,18]

Conclusion

This study assessed the absorption of a concentrated heme iron issued from the enzyme hydrolysis of bovine hemoglobin. The Ussing chamber model allowed differentiating between the two steps of absorption of iron. The net absorption of heme iron was not different from the control (Gluc). The processes of uptake and enterocyte transfer of heme iron were mainly energy dependent and, therefore, should be submitted to an active regulation, whereas half of the uptake of inorganic iron occurred by passive pathways.

Acknowledgments

We wish to express our acknowledgments to Compagnie Générale de Diététique (14050-Caen-France) for financial support. The first author is the recipient of a fund by the Conseil Régional de Basse-Normandie.

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