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Iron absorption from concentrated hemoglobin hydrolysate by rat

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Abstract

Although heme iron is highly bioavailable, the low iron content of hemoglobin prevents its use for dietary fortification; on the other hand, purified heme has low solubility and absorption rate. The present study was designed to assess the interactions between concentrated heme iron and peptides released during globin hydrolysis and cysteine and their relation with iron absorption.

Hemoglobin was hydrolyzed by pepsin or subtilisin, and then, heme iron was concentrated by ultrafiltration. Iron absorption was studied in a Ussing chamber; gluconate was used as control. Iron uptake from nonconcentrated pepsin hydrolysate and gluconate was lower than from other groups. Cysteine significantly enhanced iron uptake except from the concentrated subtilisin hydrolysate. There was no significant difference between cysteine-supplemented groups.

According to the different hydrolysis pathways of enzymes, it is assumed that the presence of hydrophobic peptides and the strength of heme–peptide interactions are both determining factors of heme iron absorption. These interactions occur mainly before iron uptake, as emphasized by the effect of cysteine.

Keywords: Heme iron; Absorption; Rat; Cysteine

1. Introduction

Iron absorption uses specific transport pathways that are localized at the upper part of the intestine; nonheme iron absorption is impaired by alkaline pH and by interactions with food components, mainly other minerals, trace elements and fibers; it shares the same membrane carrier with other divalent cations. By contrast. the absorption of heme iron uses a specific receptor. Contrary to nonheme iron, it is soluble at the duodenal pH and escapes the above-mentioned interactions with food; therefore, it is more efficiently absorbed from meals than nonheme iron (5–35% vs. 2–10%) [1]. In addition, iron salts are not well tolerated by patients and cause digestive side effects [2]. Hemoglobin has already been used with success for food fortification[3–7], but its low iron content (0.35%) limits its use in this native form. However, heme iron absorption also depends on digestive interactions because purified heme forms large insoluble polymers that are poorly absorbed [6,8–10].

Thus, heme iron absorption depends less on interactions with food than on the presence of the peptides produced by the hydrolysis of globin. These interactions must keep it in a soluble state, whatever the pH, and be weak enough to release it at the vicinity of the apical membrane [11].

The present study was designed to characterize these interactions and their relationships with iron absorption.

In the first experiment, two ways of enzyme hydrolysis, using either pepsin or subtilisin, were compared. Hemoglobin was hydrolyzed to the level of 11%, which was previously shown to ensure a good iron absorption rate [12]; then, heme concentration was increased by discarding hydrophilic peptides, which are not involved in heme–peptide interactions.

In the second experiment, we added cysteine, which is known to prevent the formation of heme polymers and enhance iron absorption [9,10,12].

The model used to assess the different steps of iron absorption was the Ussing chamber [13,14]. It uses live fully organized digestive membranes, including the mucus

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layer, which affects the diffusion of iron from the lumen to the enterocyte [15]. Although it is known that rats do not absorb heme iron as efficiently as humans do, the mechanisms of heme iron absorption are the same in both species [16–23].

2. Methods and materials

2.1. Animals

Adult female Sprague–Dawley rats, 200–250 g in weight, were obtained from IFFA CREDO (France). 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 concentrations were determined by drawing 1 ml of blood from the retroocular capillary before the experiment.

Ten groups (six animals per group) were studied. In two experimental groups, Fe was provided as a hydrolysate obtained by pepsin and subtilisin digestion of hemoglobin; in two other groups, heme iron was concentrated by ultrafiltration; these four hydrolysates were tested in presence of cysteine. Two control groups were fed gluconate iron alone or in presence of cysteine.

2.2. Preparation of the heme peptides hydrolysates

Hydrolysates were prepared at the Laboratoire de Technologie des Substances Naturelles (Lille University, France) as previously described [24].

2.2.1. Preparation of hemoglobin hemolysate

Bovine hemoglobin was prepared from centrifuged red cells provided by Veos Novo (Zwevezele, Belgium). Hemoglobin solution was adjusted to 5 g/100 g with 10 mM HCl. Hemoglobin concentration was determined according to the cyanmethemoglobin method [25]. pH was then adjusted to the appropriate level with molar HCl or NaOH.

2.2.2. Hemoglobin hydrolysis

Pepsin hydrolysis was carried out at pH 3 and at 40°C by addition of porcine pepsin (pepsin A, EC 3.4.23.1, from porcine stomach mucosa, Sigma, St. Louis, MO) with an enzyme/protein ratio of 6 g/100 g. 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. Alcalase hydrolysis (subtilisin Carlsberg, EC 3.4.21.14, Gist Brocades, Seclin, France) was performed at pH 10 and 40°C with an enzyme/protein ratio of 0.6 g/100 g. The enzyme was inactivated by addition of phenylmethylsulfonylfluoride (Sigma, St. Louis, MO) (5 μl at 100 mM in 2 ml of hydrolysate).

The digestion by the two enzymes was carried out until 11% hemoglobin was hydrolyzed. The degree of hydrolysis, defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds, was determined by the Alder-Nissen method [26].

Heme was concentrated by ultrafiltration. The resulting concentrate of heme and peptides was spray-dried to produce a water-soluble powder containing 96% dry matter.

2.3. Study of heme–peptide interactions

The strength of heme–peptide interactions in the hydrolysates was studied by adjusting their pH to 2 by addition of 1 M HCl. The solutions were then shaken and centrifuged at 5000 rpm for 15 min. Heme concentration of each supernatant was determined by the pyridine hemochromogen method [27]. Native hemoglobin and heme monomers present one absorption band at 400 nm, the Soret band [28]. The ultraviolet (UV) spectrum of hemoglobin hydrolysates assesses the association states of peptides in solutions. The UV absorption spectra of hemoglobin or heme–peptides hydrolysates were recorded on a lambda 5 UV/vis spectrometer (Perkin-Elmer) at 340–500 nm of wavelength. Twenty-five microliters of hemoglobin solution or hydrolysates were diluted with 5 ml of 50 mM phosphate buffer (pH 7.5) [24].

2.4. Ussing chamber

The diffusion cell (Marty Technologies, Marcilly-sur-Eure, France) is made of two acrylic half-cells separated by the digestive membrane (1.2 cm^2 area); each half-cell contains 3 ml.

Before the experiments, cells were washed with dilute (1/100) nitric acid (Merk Laboratories, Nogent-sur-Marne) to prevent contamination.

Ringer Lavoisier solution (Laboratoires Chaix et Marais) was used for luminal and serosal solutions. It is composed of Na^+ (139 mEq/L), K^+ (2.7 mEq/L), Ca^{2+} (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 an adequate amount of hydrolysate to obtain 100 μM iron. Iron Gluc ($C_{12}H_{22}FeO_{14}$, 2 H_2O) was purchased from Merk Laboratories. L-Cysteine (Sigma, Saint Quentin-Fallavier, France) was added as required with a cysteine/iron ratio (w/w)=105 [9,10]. D-Glucose (20 mM) was added to the serosal medium to help maintaining 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 [13,14]. The pH was adjusted to 7–7.5 by addition of chlorhydric acid to maintain both forms of iron soluble [29]. Solutes were circulated by 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 intestinal segment were controlled by histological preparation and by measuring the electrical resistance (in ohms) across the membrane (Millicell-ERS, Millipore) during the experiment. The resistance of media in the diffusion chamber (without intestinal segment) was subtracted from the values obtained

Table 1
Iron concentration and biochemical characteristics of the different hemoglobin hydrolysates

	Enzyme			
	Pepsin		Subtilisin	
Hydrolysate	Concentrated		Concentrated	
Iron concentration (g/100g)	0.30±0.01[a]	0.56±0.02	0.31±0.01	0.74±0.01
Iron concentration factor	2.17		2.32	
Soluble heme at pH 2 (%)	19.7–20.6[b]	59.5–62.5	79–80	72–78
Relative increase of Soret bandwidth	262–266	260–265	173–180	176–178

[a] Mean ± SD.
[b] Data from two assays.

in presence of intestine. The absorptive surface area (1.2 cm^2) was utilized to express the values in ohms per square centimeters. The temperature of the diffusion cells and of the media was maintained at 37°C.

2.5. Experimental design and sample analysis

The protocol used was previously described [12,14]. The animals were not fed the night before the experiment. They were killed by intracardiac injection of pentobarbital (Doléthal, Vétoquinol). A midline incision was made to expose the intestine. A 2-cm segment of duodenum, immediately distal to the first 2 cm measured from the pylorus, was removed and washed in oxygenated Ringer Lavoisier solution. The first proximal centimeter of duodenum was opened along the 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. The distal fragment of duodenum was used to measure the basal iron content of the membrane.

Luminal and serosal media were analysed before and at the end of the experiment. At the end of the experiment (2 h), cells were rinsed with diluted nitric acid until no iron could be desorbed from cell walls or duodenal tissue. The integrity of the mucosa was checked by histology (results not shown).

Pieces of duodenum were dried in an oven until their weight was stable and were digested by incubating with 1 ml nitric acid (65%) for 24 h.

Iron concentration was measured in luminal and serosal media and in duodenal tissues by atomic absorption spectrometry (Perkin Elmer 1100B).

The following values were calculated:

%Tot iron removed from the luminal medium during the experiment vs. initial iron amount

%S Iron transferred across mucosa to the serosal medium during the experiment vs. initial iron amount

$\%Q_S/Q_{Tot}$ percentage of transported iron to the serosal compartment vs. total up taken iron

Table 2
Transepithelial electrical resistance (Ω/cm^2; mean±SD; $n=6$/group) of intestinal segment throughout the experiment

Group	Minutes		
	30	75	120
Subtilisin	26.0±7.5[a]	24.0±5.4	23.5±4.7
Subtilisin, concentrated	25.3±8.0	21.3±6.5	19.6±5.2
Subtilisin+cysteine	27.1±4.7	24.6±6.9	22.0±6.3
Subtilisin, concentrated+cysteine	28.6±8.0	24.9±5.6	22.9±7.5
Pepsin	22.9±1.7	21.5±2.4	21.0±1.9
Pepsin, concentrated	28.9±5.2	26.4±7.4	23.8±8.8
Pepsin+cysteine	26.9±7.5	22.8±6.3	21.2±8.0
Pepsin, concentrated+cysteine	28.0±8.0	27.9±5.8	26.3±8.5
Iron gluconate	28.2±3.2	27.2±4.1	20.0±3.8
Iron gluconate+cysteine	31.9±5.5	27.9±5.7	23.3±3.3

No significant change (repeated measures ANOVA) was observed throughout the experience or between groups.
[a] ohm/cm^2; mean±SD; n=6/group.

2.6. Statistical analyses

Results were expressed as mean±1 SD. The changes in transepithelial resistance were analyzed by repeated measures ANOVA. The effect of concentration was assessed by ANOVA and post hoc analysis using Fisher's Exact Test. Cysteine-supplemented groups were compared with basal ones by Student's t tests; the level of significance was set at $P<.05$.

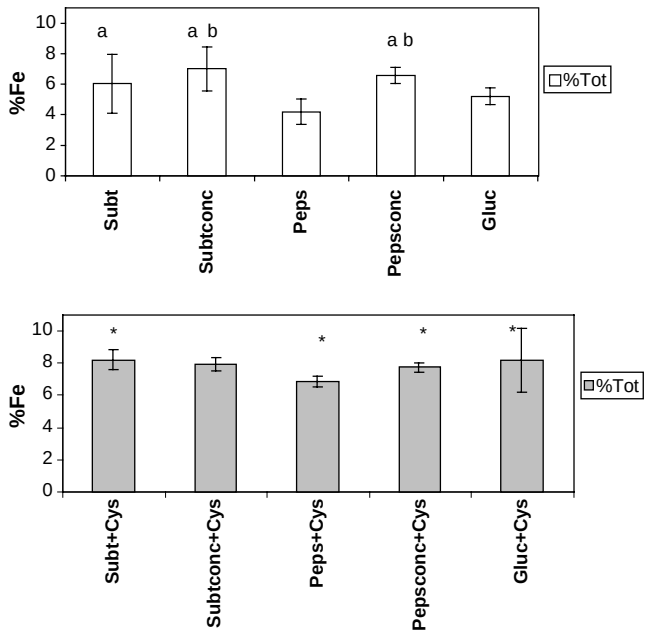

Fig. 1. Iron uptake by gut mucosa (percent removed from the luminal medium; mean±SD; $n=6$/group; initial iron luminal concentration: 100 μM). Subt=subtilisin hydrolysate of hemoglobin; Peps=pepsin hydrolysate; Subt-conc=concentrated sample of subtilisin hydrolysate; Peps-conc=concentrated sample of pepsin hydrolysate; Gluc=gluconate iron; Cys=cysteine. ANOVA between groups in the same row, followed by Fisher's Exact Test: ANOVA, $P<.007$; [a]different from Peps, $P<.03$; [b]different from Gluc, $P<.04$; Student's t test between groups with and without addition of cysteine; *different from the control without Cys ($P<.05$).

3. Results

There was no difference between hemoglobin concentrations of the experimental groups (14.3±0.5 g/dl).

The biochemical characteristics of heme–peptide complexes are given in Table 1. The solubility of hydrolysates at pH 2 indicates the strength of heme–peptides binding: increasing the concentration of the pepsin hydrolysate raised its solubility, and no change was observed for subtilisin hydrolysate. Polymerization of heme, assessed by changes in Soret bandwidth, was increased by hemoglobin hydrolysis; no further changes were observed at higher concentrations.

The integrity and viability of intestine assessed by transepithelial electrical resistance did not significantly change throughout the experience (Table 2); no significant difference was observed between groups.

Iron uptake is given in Fig. 1. In groups without cysteine addition, iron uptake from nonconcentrated pepsin hydrolysate and gluconate was lower than from other groups.

Cysteine addition significantly enhanced iron uptake from every heme and nonheme iron sources except subtilisin concentrate. There was no significant difference between cysteine-supplemented groups.

Fig. 2 gives the amount of iron transferred through the intestinal mucosa to the serosal compartment. Without cysteine, pepsin hydrolysate displayed significantly lower values than concentrated samples (pepsin and subtilisin hydrolysates) and gluconate groups.

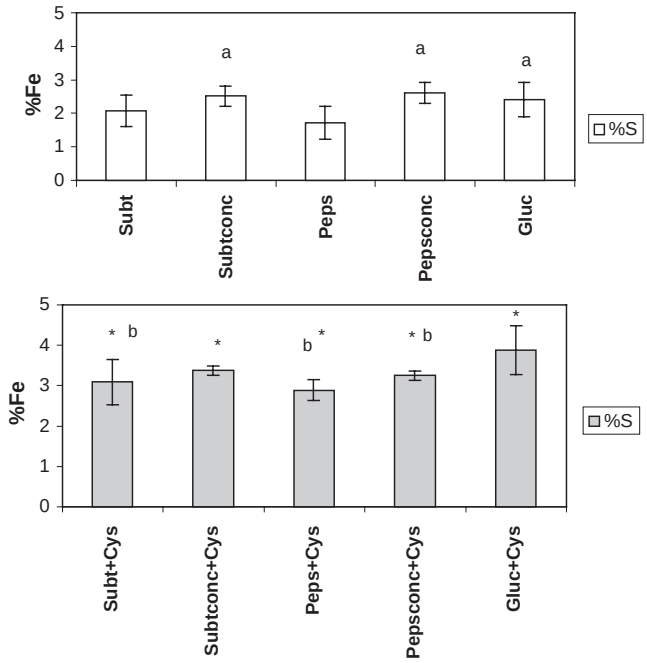

Fig. 2. Iron transferred to serosal medium (percent initial luminal medium amount; mean±SD; $n=6$/group; initial iron luminal concentration: 100 μM). See Fig. 1 for the legend. ANOVA between groups in the same row, followed by Fisher's Exact Test: ANOVA, $P<.025$; [a]different from Peps, $P<.02$; [b]different from Gluc+Cys, $P<.002$. Student's t test between groups with or without addition of cysteine: *different from the control without Cys, $P<.05$.

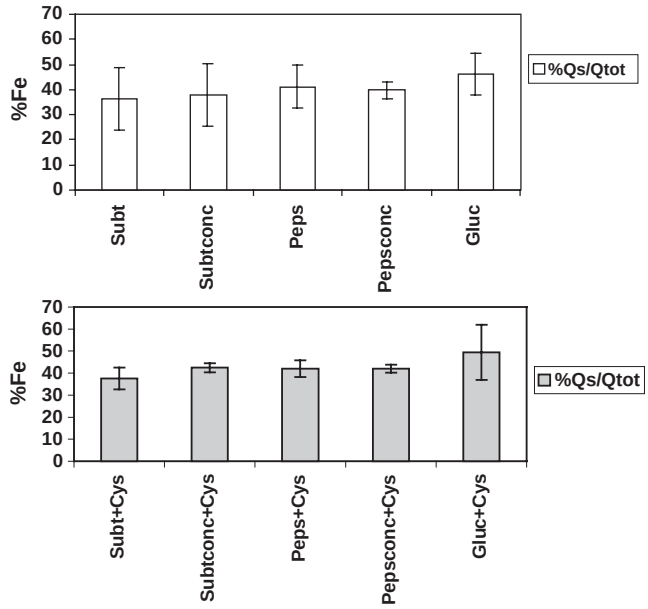

Fig. 3. Ratio of iron serosal transfer to uptake (mean±SD; $n=6$/group; initial iron luminal concentration: 100 μM). See Fig. 1 for the legend. ANOVA between groups in the same row, followed by Fisher's Exact Test: Student's t test was used to compare each sample with or without addition of cysteine. No significant difference was observed between groups.

Cysteine significantly enhanced iron transfer for all groups. When the transfer was expressed as a ratio of uptake, however (Q_S/Q_{Tot} ratio), no difference was found between groups, whatever the source of iron or the addition of cysteine (Fig. 3).

4. Discussion

The low and unpredictable absorption rate of inorganic iron has led to the search for efficient protected forms of iron. Hemoglobin iron is known to be naturally protected from digestive interactions [1,29]; however, attempts to purify heme and to increase heme iron concentration usually decrease its digestive solubility and absorption rate, which depends on the presence of peptides and amino acids produced by globin or muscle hydrolysis [8,11,12,30,31].

The present study aimed at characterizing the interactions between peptides and heme iron when they form soluble complexes.

It assessed the digestive uptake and transfer of two concentrated forms of heme produced by enzyme hydrolysis of hemoglobin and ultrafiltration of heme–peptide complexes. The membrane uptake of heme iron was similar (pepsin hydrolysates) or higher (subtilisin hydrolysates) than gluconate iron.

Cysteine enhanced iron uptake in every group except concentrated subtilisin hydrolysate. The cysteine-associated increase in iron serosal transfer, which occurred in every group, was only explained by the changes in mucosal uptake. Heme concentration did not influence heme iron absorption.

The enhancement of heme iron absorption results from a balance between specific peptides and their association with heme [11,12], which can occur by different ways. Although subtilisin and pepsin hydrolysates displayed a rather similar rate of heme uptake, their biochemical interactions with heme are quite different. The peptic hydrolysis process leads to weak heme–peptide interactions and intense polymerization of the heme, resulting in a low solubility of heme at pH 2 and in a high increase of Soret bandwidth; pepsin hydrolysis proceeds according to a "zipper" mechanism, producing medium-size peptides that bind heme and are further hydrolyzed in low-molecular-weight peptides [26].

Hemoglobin hydrolysis by subtilisin releases low-molecular weight peptides throughout the reaction. Thus, at any degree of hydrolysis, subtilisin hydrolysates consist of unattached proteins that bind strongly heme monomers and small peptides. Therefore, the increase of Soret bandwidth is lower and the level of soluble heme is higher than for pepsin hydrolysate [24]. Only hydrophobic interactions are involved in heme–peptide complexes: discarding hydrophilic peptides by ultrafiltration led to a parallel increase of the stability of hydrophobic heme–peptide association in the pepsin hydrolysate, as demonstrated by the increased heme solubility at pH 2 and iron absorption.

The use of diffusion cells as experimental model helped assessing iron uptake and mucosal transfer. Heme iron is absorbed as an unchanged porphyrin ring; the uptake involves a specific heme receptor [32]. Once uptaken by the enterocyte, both heme and gluconate iron behaved similarly, confirming that after it has been cleaved from heme by the heme oxygenase, iron enters a common intracellular pool [16,29].

The addition of cysteine to the luminal compartment enhanced the uptake and transfer of both heme and nonheme iron, as shown previously [9–11,33–36]. It forms soluble complexes with nonheme iron [35] and could influence its binding to and cross-linking of mucins; these glycosylated proteins bind iron with a high affinity, keep it soluble and favor its absorption [15,37,38]. Cysteine also binds heme, prevents the formation of large insoluble polymers and favors heme iron absorption in humans [8,10,38]. The effect of cysteine was observed for both concentrated and nonconcentrated hemoglobin hydrolysates. Although cysteine enhanced both uptake and transfer of heme and nonheme iron, further analysis showed that the increased transfer resulted from changes in mucosal uptake, emphasizing the intraluminal role of the amino acid.

5. Conclusions

Both the presence of hydrophobic peptides produced by hemoglobin hydrolysis and the strength of their interactions with heme are needed to enhance heme iron absorption. Therefore, it is possible to concentrate heme iron and keep it bioavailable by discarding the hydrophilic fractions of hemoglobin hydrolysates.

Factors that prevent digestive heme or nonheme iron polymerization such as cysteine improve its absorption by enhancing its uptake.

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