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Mechanisms of absorption of caseinophosphopeptide bound iron

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Binding iron (Fe) to the 1-25 caseinophosphopeptide obtained from enzyme hydrolysis of β casein (β CPP) improves Fe bioavailability in the rat. To assess the mechanisms involved in its absorption, a perfused, vascularized duodenal rat loop model was used in controls and in Fe-deficient (bleeding of 25% blood volume) rats. Inhibitors of oxidative phosphorylation [2-4 dinitrophenol (DNP)] and/or of endocytosis [phenylarsine oxide (PAO)] were added to the perfusion solution containing 50 μM Fe as β CPP bound Fe (Fe-β CPP) or gluconate (Fe Gluc). Fe-β CPP enhanced Fe uptake, reduced mucosal storage, and improved net absorption both in controls and in deficient animals. DNP reduced uptake, mucosal storage, and net absorption by the same percentage in Fe-β CPP and Fe Gluc perfused rats in both control and Fe-deficient animals. PAO decreased uptake, mucosal storage, and net absorption of Fe-β CPP but not of Fe Gluc. At the end of the experiment Fe serum levels were increased only in Fe Gluc animals. These results confirm the improved bioavailability of β CPP bound Fe. They suggest that at least part of its absorption can occur by a different pathway than usual Fe salts. Fe-β CPP can be taken up by endocytosis and absorbed bound to amino acids or peptides. (J. Nutr. Biochem. 10:215–222, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

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Introduction

Iron (Fe) bioavailability is usually low. It is largely determined by its interactions with other nutrients. During gastric transit, low pH releases Fe from food. In the duodenum the rise of pH leads to the formation of large, insoluble polymers of $Fe^{3+}OH$. Reducing agents such as ascorbic acid or ligands such as ascorbic acid or other weak acids can keep Fe soluble until it binds to mucosal mucin.[1,2] Dietary proteins are likely to enhance Fe solubility when they increase its in vitro solubility and dialyzability.[3–5] Whole proteins are assumed to be too large to diffuse across mucin filter.[6] Therefore, they could release Fe too far in the intestine for efficient absorption.

Phosphoproteins present clusters of phosphorylated serine that bind calcium and other cations such as zinc and Fe[7–11]; however, covalent bindings developed between Fe and phosphoserine are stronger (approximately 100 times) than ionic binding of zinc or calcium.[12–14] In addition, Fe complexes are not dissociated by variations of pH. Although they keep Fe soluble, they could be too large to improve its absorption.[6] In fact, Hurrell et al.[15] showed that the low absorption rate of Fe supplied with milk proteins is improved by their enzyme hydrolysis. Enzyme digestion of caseins yields low molecular weight caseinophosphopeptides, which keep cations in a soluble form.[12,16,17]

Therefore, it was assumed that binding Fe to these peptides could improve its digestive absorption. Some conflicting results were reported on the effects of caseinophosphopeptides on calcium and zinc absorption.[16,18–21] However, it should be kept in mind that these caseinophosphopeptides were not usually well characterized and that they form only weak complexes with cations other than Fe.[7,9,10]

On the contrary, we used the precisely defined 1-25 caseinophosphopeptide of β-casein (β CPP), which contains four of the five phosphoserine residues of the native β

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casein and can bind four atoms of Fe.[12] Preliminary reports on Fe metabolism showed that binding Fe to β CPP improves its bioavailability in young deficient rats[22] and increases its absorption by the isolated, vascularized duodenal rat loop model.[23] These results could not be explained only by an increased digestive solubility. The strong affinity of caseinophosphopeptides for Fe[12,24] and the occurrence of biologically active peptides issued from milk proteins in plasma[25–27] suggest that β CPP bound Fe could be absorbed by a different pathway other than non-heme Fe.

The present study was designed to assess the mechanisms involved in the mucosal step of β CPP bound Fe absorption. For that purpose, an isolated duodenal rat loop was used. The absorption of β CPP bound Fe (Fe-β CPP) and Fe gluconate (Fe Gluc) was compared in normal and Fe-deficient rats. Fe gluconate was chosen because of its well-known good bioavailability and because it remains soluble at alkaline pH.[28] The experiments were done in the presence of inhibitors of endocytosis [phenylarsine oxide (PAO)] and oxidative phosphorylation [2-4 dinitrophenol (DNP)] in order to study the apical membrane uptake of Fe and to differentiate between passive and active pathways involved in its transfer across mucosa.

Materials and methods

Preparation of 1-25 caseinophosphopeptide of β casein

β Casein was isolated from industrially-made sodium caseinate (Armor Protéines, Saint-Brice-en-Coglès, France) by cold solubilization (pH 4.5; 4°C), followed by ion exchange chromatography.[10,12]

Then, 1-25 caseinophosphopeptide β casein was obtained by tryptic digestion of β casein, and calcium/ethanol precipitation of the β CPP.[28] Fe was bound to β CPP by adding $FeCl_2$ solution [4.10^{-2} M with tenfold excess of $FeCl_2$ solution; pH 5.3; 30 minutes; 25°C (Milli Q system, MILLIPORE, Paris, France)]. Unbound Fe was discarded by ultracentrifugation and dialfiltration on a regenerated cellulose membrane with a cut off of 3,000 Da (membrane SIOY3, Amicon, Lexington, MA USA). The Fe-β CPP complex obtained was then freeze-dried. Chelated Fe and residual calcium were measured by atomic absorption spectrometry (Varian AA 1275 VARIAN, Paris, France). As expected these assays showed that 1 mole of peptide binds 4 moles of Fe. A control without β CPP was incubated and dialyzed under the same conditions.

Animals

Adult Sprague-Dawley rats weighing 250 to 300 g were fed with a semi-synthetic feeding for adult rats providing 200 g protein as casein and 200 mg Fe/kg diet (UAR, Villemoisson-sur-Orge, France). Animals had been starved for 12 hours before study but had free access to water.

A total of 16 groups ($N = 6$ by group) was studied: 8 groups of normal, nondeficient animals and 8 groups of deficient animals were perfused with one of the two forms of Fe [Fe gluconate (Fe Gluc) or Fe-β CPP]. Experiments were performed without (controls) or with the addition of one of the inhibitory agents (DNP, PAO) or of both.

Acute Fe deficiency was obtained by drawing 25% blood volume by two retroorbital punctures at 24-hour intervals within the 48 hours prior to the experiment.

Perfusion solutions

The composition of perfusion solution was adapted from Ringer-Lavoisier solution. Its pH was 4.5 (adjusted to proximal duodenum pH). It was isotonic to the plasma (285–300 mOsm) and contained 50 μM Fe as gluconate or Fe-β CPP. When necessary DNP and PAO were added at concentrations of 500 μM and 100 μM, respectively.

Experimental design

Anesthesia was performed with ketamine (Kétalar™, PANPHARMA, Fougerès, France), which has no effect on digestive motility. Blood was drawn by a retroorbital puncture for a blood cell count and assay of serum Fe. The duodenum was exposed by laparotomy. It was perfused through a catheter inserted into the pylorus. The effluent was collected at the angle of Treitz. Every element of the perfusion device had been previously washed with a solution of Triton ×100 (1 g/L) to prevent any contamination. The perfusion solution was kept at 37°C by thermostatic control and was delivered at 0.16 mL/min, using a peristaltic pump to avoid loop distention. The perfusion lasted for 2 hours. A nonabsorbable marker (polyethylene glycol 4000, PROLABO, Paris, France) was added to assess actual net water fluxes. After 2 hours of perfusion, another retroorbital puncture was performed to measure serum Fe; then the animal was euthanized by an overdose of Doléthal™ (VETOQUINOL, Lure, France); the perfused loop was washed with saline, withdrawn, and dried in a oven until a steady weight was obtained.

Sample analysis

Fe was measured by atomic spectrometric absorption (Perkin-Elmer 3030 PERKIN-ELMER, Courtoboeuf, France) in the serum, perfusion solution, digestive effluent, and mucosa of the perfused segment. Tissues were digested in nitric acid at ambient temperature during 24 hours. Ringer-Lavoisier solution was used as a blank.

Polyethyleneglycol (PEG) was measured in the perfusion solution and the digestive effluent by a turbidimetric method described by Hydén.[30] Fe disappearance from digestive lumen (Q1:μmol) was calculated as follows:

$$Q1 = (1 - \frac{[PEG]_T}{[PEG]_e} * \frac{[Fe]_e}{[Fe]_T)} * D * t * [Fe]_T$$

where [PEG] and [Fe] represent PEG and Fe concentrations in perfusion solution (T) and in the effluent (e), respectively. D and t are the delivery rate (mL/min) and the time of collection.

Fe stored by the mucosa (Q2:μmol) during the perfusion was calculated as follows:

$$Q2 = ([Fe]_m - [Fe]_{m0}) * P_m$$

where m is the segment of perfused intestinal mucosa and P_m its dry weight; $[Fe]_{m0}$ is the mean Fe concentration displayed by the duodenal mucosa of a separate group of 24 rats previously perfused for 2 hours with a Fe free solution. Net Fe absorption (Fe abs:μmol) during the perfusion was Fe abs = Q1 − Q2.

Statistics

Results are expressed as mean and standard deviations. Groups were compared by two-way analysis of variance (ANOVA) and Student's *t*-test.

Table 1 Digestive absorption of iron (Fe) provided as β CPP (Fe-β CPP) and gluconate (Fe Gluc) by isolated, vascularized rat loop model in Fe-deficient or normal rats

	Normal		Fe Deficient		
	Fe Gluc	Fe-β CPP	Fe Gluc	Fe-β CPP	ANOVA
N	6	6	6	6	
Q1	51.3 ± 1.9*	64.2 ± 1.1[a,b]	60.9 ± 2.9[a,b]	70.5 ± 0.6[a]	$P < 0.001$
Q2	18.7 ± 2.2	15.2 ± 1.9[a,b]	11.8 ± 2.5[a,b]	15.0 ± 3.4[a]	$P < 0.001$
Fe^{Abs}	32.6 ± 3.3	49.0 ± 2.1[a,b]	49.1 ± 4.6[a,b]	55.6 ± 3.4[a]	$P < 0.001$

*% Moles perfused; mean ± 1 SD; initial iron concentration of the perfusion solution: 50 μM (Fe Gluc or Fe-β CPP); Q1: iron disappeared from digestive lumen; Q2: iron stored by the mucosa; Fe^{Ab}: absorbed iron.
[a]Different ($P < 0.05$) from normal group Fe Gluc.
[b]Different ($P < 0.05$) from deficient group Fe-β CPP.
β-CPP–1-25 caseinophosphopeptide obtained from enzyme hydrolysis of β-casein. ANOVA–analysis of variance.

Results

Initial hematologic data

An ANOVA comparing the initial values of red blood cell counts (hemoglobin concentration and hematocrit) did not show any significant difference between the eight normal groups or between the eight Fe-deficient groups: $F = 1.06$ ($P = 0.40$) and $F = 0.92$ ($P = 0.39$) for hemoglobin levels; $F = 1.08$ ($P = 0.39$) and $F = 1.76$ ($P = 0.12$) for hematocrits. Therefore, the eight normal and Fe-deficient groups were pooled to assess the effect of bleeding. Mean hemoglobin (±1SD) was 14.1 ± 0.2 and 10.4 ± 0.2 for normal and Fe-deficient groups (Student's *t*-test, $t = 93.9$, $P < 0.0001$); mean hematocrit was 39.6 ± 0.2 and 29.5 ± 0.3 for normal and Fe-deficient groups (Student's *t*-test, $t = 190.9$, $P < 0.0001$).

Influence of Fe status and form of perfused Fe on its absorption

Results are displayed in *Table 1*. Net Fe absorption was higher in Fe-β CPP animals than in Fe Gluc animals, and higher in Fe-deficient rats than in control rats ($P < 0.001$). This is due to same differences in disappearance from digestive lumen ($P < 0.001$) and opposite trends in mucosal retention ($P < 0.001$).

Influence of DNP

In nondeficient groups, DNP inhibited by 50 to 60% the disappearance, by 40% the mucosal retention, and by 65% the net absorption of Fe compared with control animals (*Figure 1*). No differences were observed between Fe Gluc and Fe-β CPP rats ($P < 0.001$). Similar results were obtained in Fe-deficient rats (*Figure 2*).

As a result, 11.4% and 7.5% Fe were absorbed by a non-active pathway in Fe Gluc and Fe-β CPP rats, respectively (*Figure 3*).

Influence of PAO

A significant inhibition of disappearance, mucosal retention, and net absorption of Fe was observed in Fe-β CPP rats only. No effect of PAO was observed in Fe Gluc perfused animals.

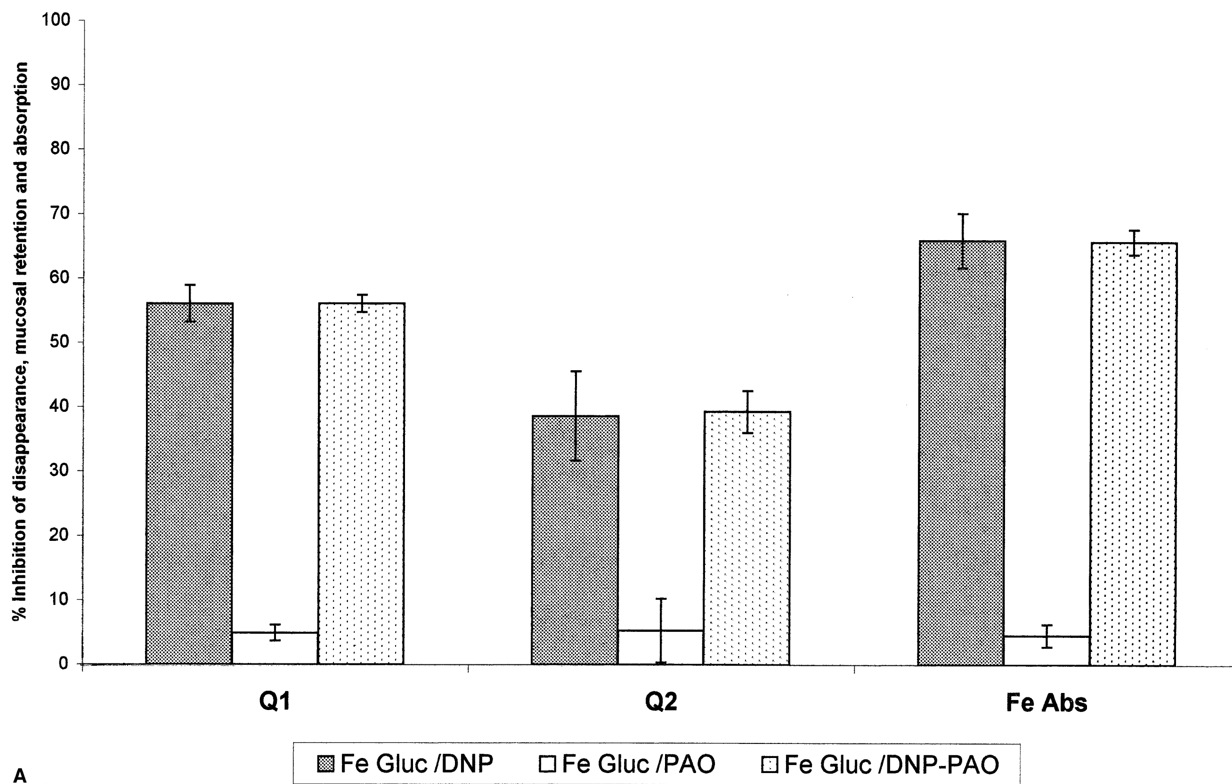
Simultaneous perfusion of DNP and PAO resulted in an inhibition of Fe disappearance, mucosal retention, and net absorption equivalent to the sum of their individual effects (*Figure 1* and *Figure 2*).

Serum Fe

Results are displayed in *Table 2*. At T1, ANOVA did not display any difference between the eight normal groups or between the eight Fe-deficient groups, and a subsequent comparison between the pooled results of normal and Fe-deficient T1 serum showed a significant effect of bleeding ($P < 0.0001$). A significant increase between the beginning and the end of the experiment was observed only in Fe Gluc rats, either control ($P < 0.005$) or Fe deficient ($P < 0.005$; Student's *t*-test). This difference was still observed after PAO in these two groups ($P < 0.005$). No change was observed with DNP in control animals and Fe-β CPP rats.

Discussion

Nutrient interactions have a great influence on Fe bioavailability. In particular, milk components such as calcium and proteins impair its absorption.[15,16,30,31] Phosphoproteins such as caseins are strong ligands for Fe due to ionic and coordination bonds,[9,11,12,32] which could release Fe too far in the digestive tract to be absorbed; in addition to this, during the formation of complexes Fe is oxidized into ferric ion,[11,13] which is usually poorly absorbed because it becomes insoluble in digestive lumen at alkaline pH.[33] Dialyzability, and not only solubility, is predictive of protein induced absorption of Fe[3,6] because whole proteins, and therefore bound cations, are assumed to be too large to diffuse across mucin.[6] Indeed, although Fe solubility in the digestive tract is increased by presence of caseins,[9,12] it is poorly absorbed, but this absorption is improved when milk proteins are hydrolyzed.[15] Therefore, binding Fe to low molecular weight phosphopeptides issued from enzyme hydrolysis of casein should improve its digestive solubility and its absorption as well. Previous experimental studies in the young Fe-deficient rat showed that Fe bound to the purified β CPP has a better bioavailability than the reference salt ($FeSO_4$) or Fe bound to or in presence of whole β casein.[22] Despite an improvement in tissue storage and hemoglobin levels, this study was unable to display any difference in Fe absorption rate. However the metabolic balance was performed at the end of the first week of repletion, whereas in Fe-deficient rats Fe absorption seems to be increased only during the first 3 days of repletion.[34] When Fe absorption is studied by the isolated, vascularized duodenal rat loop within 2 days after acute bleeding, β CPP bound Fe displays a better



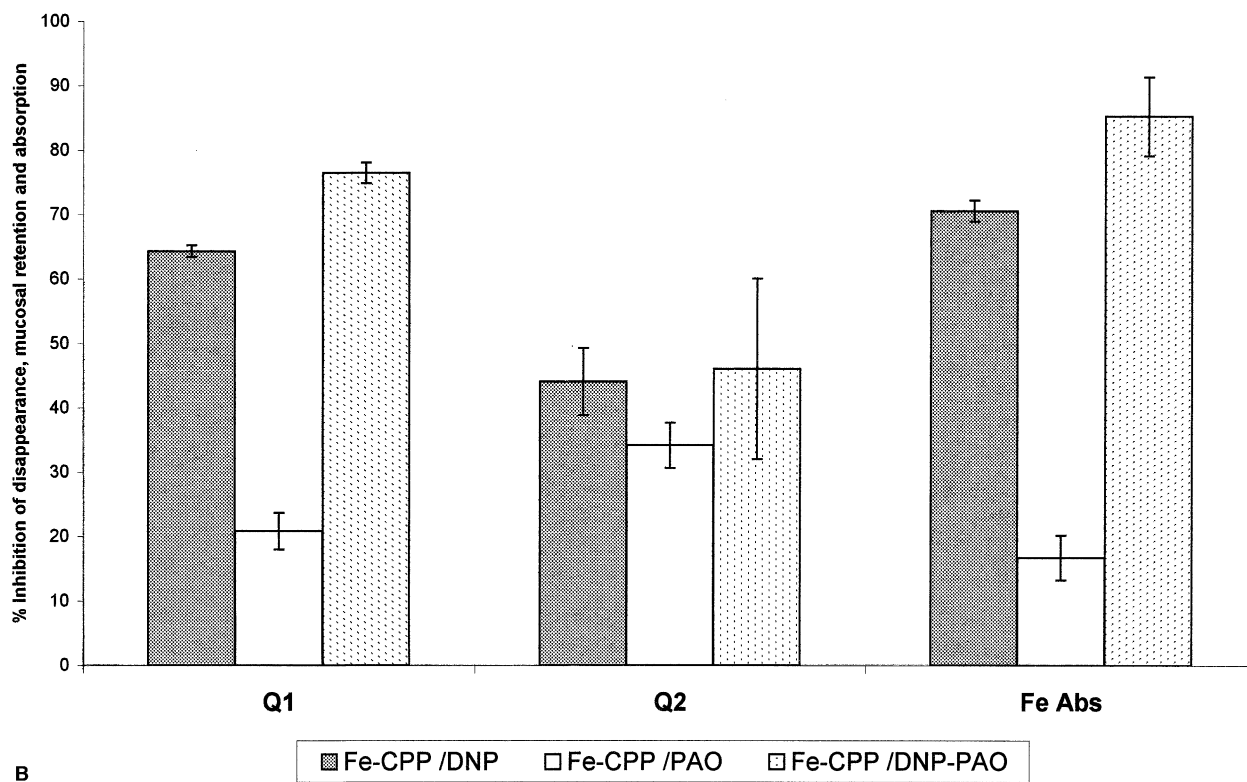


Figure 1 Inhibition of iron (Fe) absorption by 2-4 dinitrophenol (DNP) and/or phenylarsine oxide (PAO) in normal, nondeficient rats. Fe concentration of perfusion solution in every group is *(A)* 50 μM as iron gluconate or *(B)* β casein (β CPP) bound iron. In control groups, no inhibitor was added. An oxidative phosphorylation inhibitor (DNP 500 μM) or an endocytosis inhibitor (PAO 100 μM), or both (DNP-PAO) were added. %Q1, %Q2, and %Fe Abs as in *Table 1*. Inhibition of absorption is calculated from data of experiments with PAO±DNP compared with the mean value of the DNP or PAO free groups (%); residual absorption is 100% inhibition.

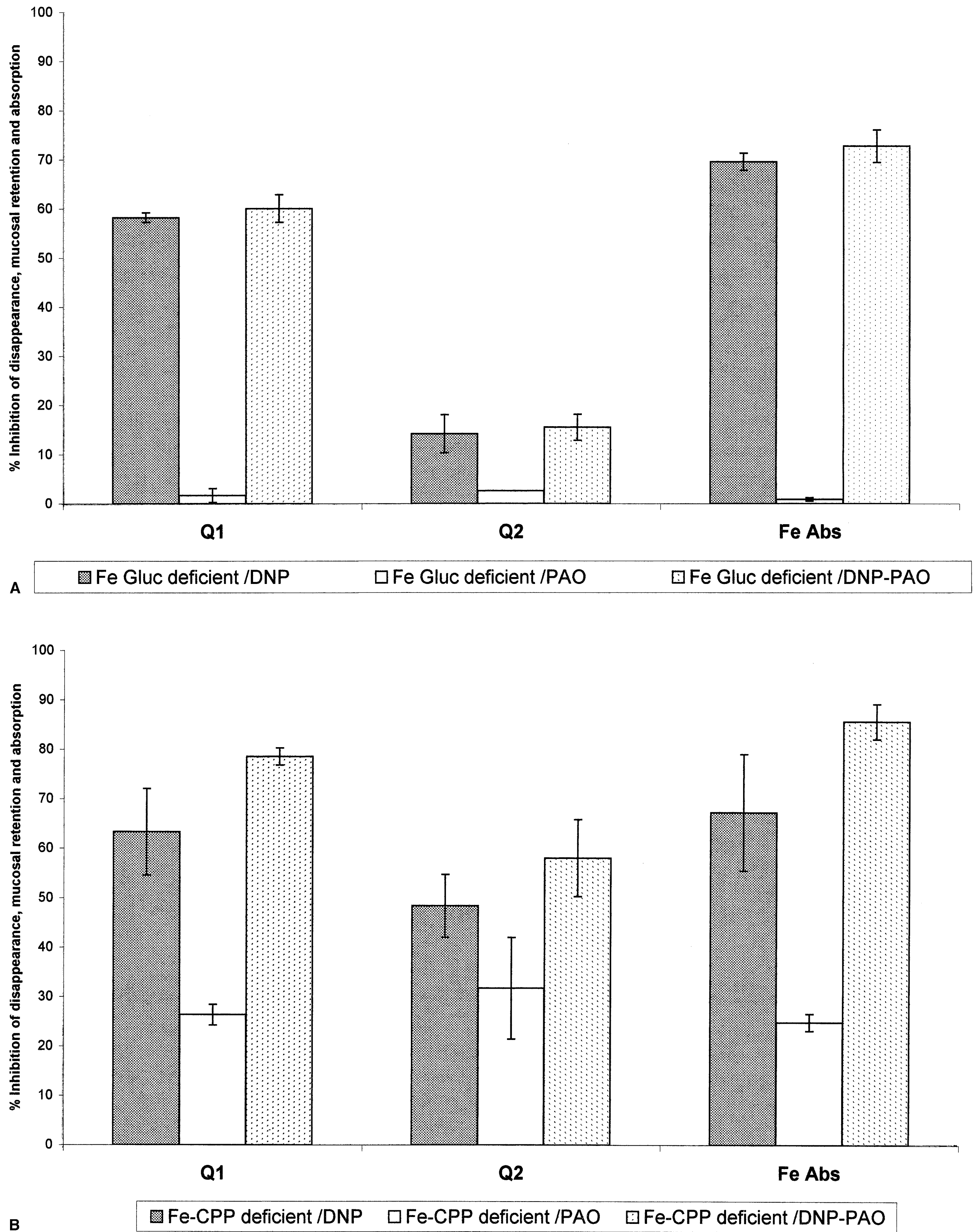


Figure 2 Inhibition of iron (Fe) absorption by 2-4 dinitrophenol (DNP), phenylarsine oxide (PAO), or DNP-PAO in Fe-deficient rats. Fe concentration of perfusion solution in every group is *(A)* 50 μM as iron gluconate or *(B)* β casein (β CPP) bound iron. In control groups, no inhibitor was added. An oxydative phosphorylation inhibitor (DNP 500 μM) or an endocytosis inhibitor (PAO 100 μM), or both (DNP-PAO), were added. %Q1, %Q2, and %Fe Abs as in *Table 1*. Inhibition of absorption is calculated from data of experiments with PAO±DNP compared with the mean value of the DNP or PAO free groups (%); residual absorption is 100% inhibition.

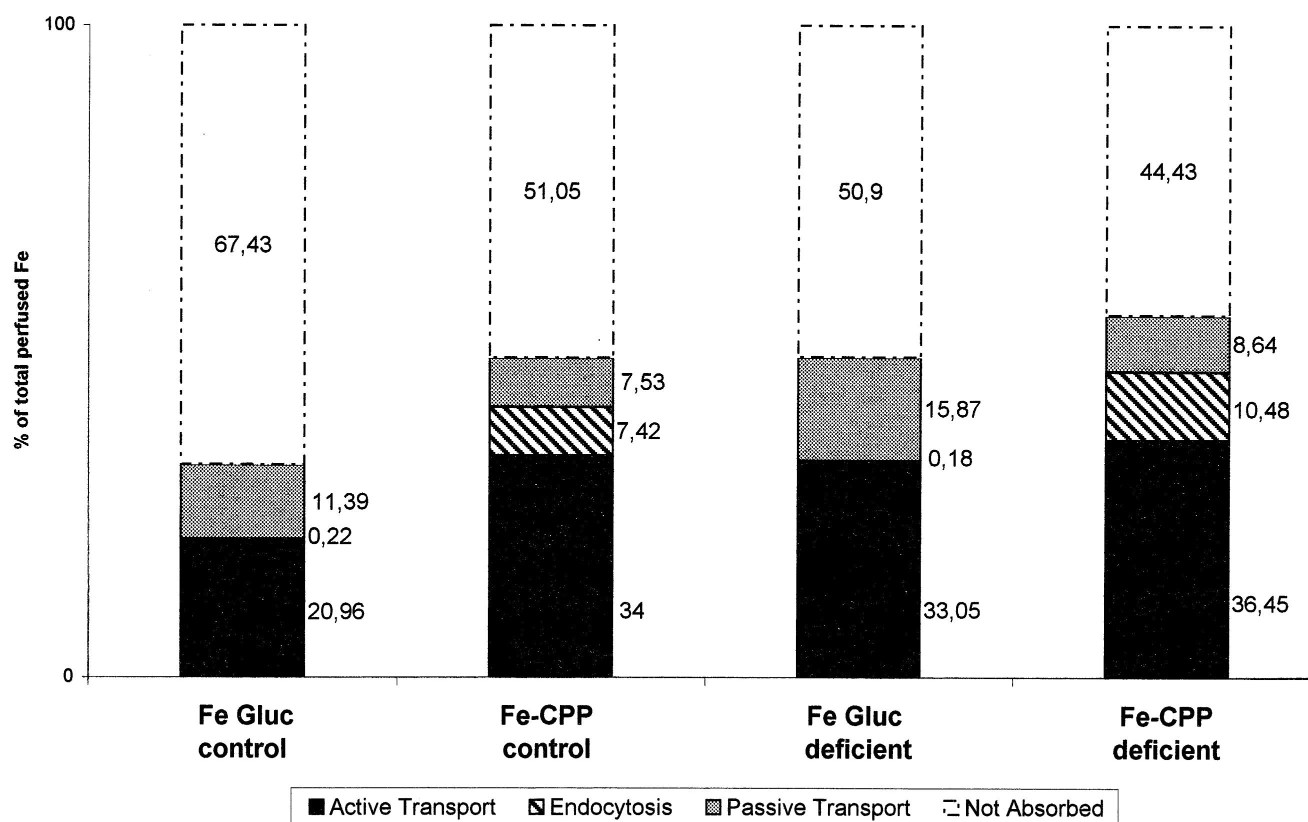


Figure 3 Relative contribution of respective pathways to iron (Fe) absorption according to its source and Fe status of animals. Absorption due to active or passive transport or endocytosis was calculated from data of *Table 1* and from *Figure 1* and *Figure 2*. Fe concentration of perfusion solution in every group is 50 μM as iron gluconate or β casein (β CPP) bound iron. In control groups, no inhibitor was added. An oxydative phosphorylation inhibitor [2-4 dinitrophenol (DNP) 500 μM] or an endocytosis inhibitor [phenylarsine oxide (PAO) 100 μM], or both (DNP-PAO), were added. %Q1, %Q2, and %Fe Abs as in *Table 1*. Inhibition of absorption is calculated from data of experiments with PAO±DNP compared with the mean value of the DNP or PAO free groups (%); residual absorption is 100% inhibition.

absorption rate than Fe Gluc and decreases the inhibitory effect of calcium.[23] Fe concentrations used in this work, however, were higher than usual dietary levels.

The present study was designed to confirm the enhancing effect of binding Fe to β CPP, using the same experimental model at lower digestive Fe concentrations, and to assess

Table 2 Serum iron variations during duodenal rat loop perfusion with iron (Fe) bound to the β caseinophosphopeptide (Fe-β CPP) or Fe gluconate (Fe Gluc) in normal and Fe-deficient animals and influence of DNP or/and PAO addition to the perfusion

	Normal		Deficient	
	T1	T2	T1	T2
Fe Gluc	262.8 ± 18.1*	307.5 ± 20.0[b]	135.5 ± 12.6	189.5 ± 19.5[b]
Fe-β CPP	264.6 ± 16.0	268.2 ± 13.5	137.3 ± 11.8	139.2 ± 15.4
Fe Gluc DNP	276.4 ± 20.0	285.3 ± 20.2	134.2 ± 14.3	138.1 ± 17.0
Fe-β CPP DNP	278.8 ± 25.2	280.7 ± 22.0	139.4 ± 12.1	141.1 ± 15.6
Fe Gluc PAO	267.1 ± 21.1	301.3 ± 23.1[b]	135.3 ± 14.2	179.2 ± 13.6[b]
Fe-β CPP PAO	275.1 ± 15.3	279.2 ± 19.3	139.5 ± 19.8	136.4 ± 20.0
Fe Gluc DNP-PAO	283.1 ± 21.3	281.4 ± 19.9	132.1 ± 16.5	139.8 ± 16.9
Fe-β CPP DNP-PAO	279.5 ± 19.4	286.8 ± 23.6	139.0 ± 20.0	142.2 ± 18.9
ANOVA between groups	F = 0.88 *P* = 0.53		F = 0.18 *P* = 0.99	
Mean T1	273.4 ± 19.6[a]		136.5 ± 14.5	

Iron was measured in serum before (T1) and at the end (T2) of intestinal perfusion. Composition of perfusion solutions were as in *Figure 1*.
*μg/100 mL mean ± 1 SD.
[a]Different (Student *t*-test: $P < 0.0001$) from mean deficient group.
[b]Different (Student *t*-test: $P < 0.005$) from T1.
DNP–2-4 dinitrophenol. PAO–phenylarsine oxide.

the mechanisms involved in Fe-β CPP absorption. It was assumed that some of this Fe could be absorbed in a bound form, according to the discovery of biologically active peptides issued from milk proteins in the serum of young animals and humans. This implies that they had crossed the digestive barrier.[27] In the first case, our data confirmed our previous results that at a 50 μM concentration Fe-β CPP is better absorbed than Fe Gluc and, as expected, Fe deficiency enhanced Fe absorption irrespective of its form, suggesting that Fe-β CPP absorption did not escape the regulation control. Fe-β CPP improved net absorption of Fe, suggesting that binding Fe to β CPP influenced both Fe access to apical membrane, due to an increased solubility, and Fe metabolism inside the enterocyte.

DNP is a potent inhibitor of oxidative phosphorylation. Although a short-term (5 minutes) experiment did not show any effect of DNP on Fe uptake,[35] another study used it to block efficiently the active transport of Fe.[36] This protocol does not allow differentiation between the different steps of absorption that could be inhibited by DNP during a 2-hour experiment: Absorption of Fe into the organism is energy dependent, the uptake phase being described both as energy independent and dependent.[33,37] Nevertheless, gluconate and Fe-β CPP absorption were similarly inhibited by DNP, suggesting that they share the same absorptive pathways and that the enhanced absorption of Fe-β CPP occurs through an added specific mechanism.

After energy dependent processes were abolished by DNP, 7 to 15% of Fe was still absorbed; passive absorption could be of physiologic importance because it depends on intraluminal concentrations and escapes homeostatic control; it could involve a paracellular pathway[33] or passive diffusion across the apical membrane, beside the saturable active carrier mediated transport system.[38] Because only Fe^{2+}—and not Fe^{3+}—seems to be concerned by this uptake mechanism, free ferric ion yielded from Fe-β CPP cleavage should have been reduced into a ferrous form by a membrane reductase before being taken up.[39,40] Passive absorption was slightly enhanced by Fe deficiency, perhaps as a result of a diminished intracellular concentration of Fe, such as previously reported[36,41]; however, an increase of the active component of absorption also has been described.[42] Passive Fe absorption was enhanced neither by β CPP, such as reported for calcium,[43] nor by the increased intestinal permeability induced by Fe deficiency such as has been reported in children.[44]

PAO is a ubiquitous inhibitor of endocytosis.[45–47] It had absolutely no effect on Fe Gluc absorption; cytoskeletal inhibitors such as colchicine were previously reported to affect Fe^{3+} absorption[48,49]; in fact, colchicine probably does not affect endocytosis per se, but acts rather on the intracellular migration.[50] On the other hand PAO decreased by 20 to 30% the uptake and net absorption of β CPP bound Fe, which seems to be absorbed by this specific pathway, perhaps being bound to an amino acid or a peptide. Biologically active peptides issued from enzyme digestion of casein have been found intact in plasma[26,27]; dietary peptides and proteins can be taken up by endocytosis and transported through the digestive epithelium; approximately 10% can be absorbed in an intact form.[26,51] Fe is slowly cleaved from β CPP[12]; β CPP is partially resistant to enzyme hydrolysis[12,24] and is found in feces.[7,16] Therefore, a large part of Fe-β CPP could remain intact in duodenum lumen, allowing Fe to be taken up by endocytosis, together with β CPP or any derived peptide.

The occurrence of distinct absorption pathways between Fe Gluc and Fe-β CPP also is suggested by the differences observed between changes in Fe serum levels, which increased during experiments with Fe Gluc and remained unchanged with Fe-β CPP. Plasma Fe issued from Fe-β CPP could have been cleared more efficiently than Fe Gluc. This is in agreement with the previous observation of increased tissue storage in rats fed Fe-β CPP.[22] Fe stored by rats during Fe-β CPP feeding is available for synthesis of hemoglobin, which reaches normal levels at the end of repletion, whereas $FeSO_4$ repleted animals still exhibit lower values.[22] Such a discrepancy between absorption and serum levels is observed when milk proteins are added to a test meal during a study of zinc absorption.[51] Metabolism of selenium also is influenced by this form: When it is provided as selenomethionine, its availability depends on the release of methionine by tissues.[52]

Conclusions

The present study confirms that β CPP bound Fe has a better bioavailability than inorganic salts. This could be explained by the occurrence of distinct absorption pathways: Part of Fe-β CPP was absorbed by endocytosis, in addition to the usual passive and energy dependent mechanisms. The enhanced absorption of Fe-β CPP was observed in normal and Fe-deficient animals. It is clear that further studies are needed to assess these beneficial effects in humans.

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