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Improved absorption of caseinophosphopeptide-bound iron: role of alkaline phosphatase

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Abstract

Hydrolysis of proteins could lessen their inhibiting effect on the poor absorption of cow's milk iron (Fe), which is responsible for the high incidence of Fe deficiency worldwide. When bound to Fe, caseinophosphopeptides (CPP) derived from milk proteins resist luminal digestion, enhance Fe solubility and could improve its bioavailability; brush border enzyme alkaline phosphatase activity could influence iron absorption by releasing free Fe; this study assessed its role in the absorption of CPP-bound Fe. Rat duodenal loops were perfused with Fe gluconate or Fe bound to the CPP of β casein [β-CN (1–25)], with or without the addition of an inhibitor of alkaline phosphatase, Na_2WO_4. The uptake of Fe–β-CN (1–25) was greater than Fe gluconate. Na_2WO_4 enhanced the uptake of Fe–β-CN (1–25) and not of Fe gluconate. So the release of free, insoluble Fe, by alkaline phosphatase seems to be prevented by providing Fe in the Fe–β-CN (1–25) complex form. Its good disappearance rate makes β-CN (1–25)-bound Fe a candidate for food fortification.

Keywords: Biologically active peptides; Caseinophosphopeptides; Iron absorption; Alkaline phosphatase

1. Introduction

Proteins could contribute to the poor iron (Fe) absorption from cow's milk [1,2]. Caseinophosphopeptides (CPP) are released from caseins during their digestion. Their sequences include the phosphorylated clusters of native proteins and bind iron strongly [3–6]; they are partly protected against further hydrolysis during luminal digestion, form stable complexes with iron at gastric pH and keep it soluble at intestinal pH [7,8]. In some studies, CPP enhance the absorption of CPP-bound iron [9,10], but in others, this latter one is decreased in the presence of CPP [11] and improved by CPP dephosphorylation [12].

However, enzyme hydrolysis of whole casein yields several peptides which have opposite effects on iron absorption: α_S-casein CPP display an inhibitory effect on absorption of added iron [13], while positive effects were observed for iron bound to CPP from β casein [9]. In addition, it was previously shown that iron–CPP complexes are less susceptible than free CPP to the activity of enzymes (alcalase and pancreatic proteases) [4,8], which could explain discrepancies between in vivo and in vitro studies.

As iron–CPP complexes are not hydrolysed during luminal digestion, the susceptibility of CPP and iron–CPP complexes to alkaline phosphatase is a key point for assessing the brush border metabolism and the mechanisms of absorption of complexed iron and for improving the bioavailability of cow's milk iron.

The present in vivo study was part of a program on functional milk peptides which aims at improving iron bioavailability; it assessed the susceptibility of β-CN (1–25), either free or bound to Fe, to digestion by membrane alkaline phosphatase, because resistance to hydrolysis is a good index of the functional properties of functional peptides; the role of alkaline phosphatase in the iron absorption of β-CN (1–25)-bound Fe was also studied; the model used was

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the perfused rat intestinal loop, in the presence of an inhibitor of alkaline phosphatase, Na_2WO_4 [12,14].

2. Methods

2.1. Production of the phosphopeptide β-CN (1–25)

β-CN (1–25) is the phosphorylated N-terminal fragment of β-casein of molecular mass 3124 containing four phosphoseryl residues. It chelates four iron atoms per molecule with high affinity. β-CN (1–25) and Fe–β-CN (1–25) complex were prepared as previously reported [9]. The amount of Fe bound to β-CN (1–25) was determined by atomic absorption spectrometry (Varian, Model AA 1275; 91941 Les Ulis, France). The dialysed sample was freeze-dried. Double distilled and deionized water was used. All glassware and the polyethylene tubes used for the samples were washed and rinsed in distilled water.

2.2. In vivo digestion of free and iron bound β-CN (1–25)

Adult Sprague–Dawley rats weighing 200 to 250 g were given a semisynthetic feeding for adult animals providing 200 g of protein as casein and 200 mg Fe/kg diet (UAR, Villemoisson-sur-Orge, France). They were starved for 12 h before the study but had free access to water.

Two groups were perfused with free CPP, in the presence or absence of Na_2WO_4. Four groups were perfused with one of the two forms of Fe [Fe gluconate or Fe–β-CN (1–25)]; experiments were performed without (controls) or with the addition of Na_2WO_4. The composition of perfusion solute was adapted from Ringer-Lavoisier solute: its pH was adjusted to 7 (duodenum pH); it was isotonic to plasma (285–300 osm) and contained 100 μmol/L Fe as gluconate or Fe–β-CN (1–25); this complex contains 0.22 mmol/L of β-CN (1–25) (14 mg); the same concentration of the phosphopeptide was used for perfusions of free peptide. When necessary, Na_2WO_4 was added at a concentration of 1 mM.

Anesthesia was performed with Kétamine (Kétalar, Pfizer Laboratories, 75668, Paris). The duodenum was exposed by a laparotomy; it was perfused through a catheter inserted into the pylorus; effluent was collected at the angle of Treitz. Every element of the perfusion device was previously washed with a solution of Triton ×100 (1 g/L) to prevent any contamination. The perfusion solute was kept at 37°C by thermostatic control and was delivered at 0.16 ml/min, using a peristaltic pump, to avoid loop distention. After 2 h of perfusion, the animal was killed by an overdose of Doléthal.

2.3. Analytical procedures

The hydrolysis and dephosphorylation reactions of β-CN (1–25) were monitored by electrospray mass spectrometry (ESI-MS) working online with a high-performance liquid chromatography (HPLC) system (Waters 625 LC System; Waters, Milford, MA).

Before analysis, the lumen contents were first incubated 1 h with 18 mM EDTA at 25°C in order to dissociate iron–phosphopeptide complexes. This step was added in order to improve peptide separation and detection. After centrifugation (3000×g, 10 min), the supernatants were adjusted to pH 2 with 5% trifluoroacetic acid then analyzed. Chromatographic separation of the sample components was performed at pH 2 using solvent A (0.1% trifluoroacetic acid, 5% acetonitrile in water) and solvent B (0.1% trifluoroacetic acid, 80% acetonitrile in water). The samples were introduced into the mass spectrometer (PE-Sciex API III; Sciex, Thornhill, Ontario, Canada) through a C18 symmetry column (2.1×150 mm, Waters) eluted at a flow rate of 0.25 ml/min, at 40°C, with a linear gradient of 5% to 60% solvent B in solvent A over 30 min.

Mass spectrometry analysis was performed on two animals of every group. Fe was measured by atomic spectrometric absorption (Perkin-Elmer 3030) on perfusion solute, digestive effluent and mucosa of the perfused segment. Ringer-Lavoisier solute was used as blank.

Fe disappearance from digestive lumen (Q1, μmol) was calculated as follows:

$$Q1 = (1 - ([PEG]t/[PEG]e) \times ([Fe]e/[Fe]t) \times D \times T \times [Fe]t$$

where [PEG] and [Fe] represent PEG and Fe concentrations

Table 1
Effect of Na_2WO_4 on the digestion and dephosphorylation processes of β-CN (1–25) ex vivo as a function of perfusion time

Groups	Peptide sequences derived from β-CN (1–25) detected by mass spectrometry	
	1-h perfusion	2-h perfusion
β-CN (1–25)	β (1–6); β (2–6); β (1–25); β (2–25); β (1–25)-1P; β (2–25)-1P; β (2–25)-2P; β (2–25)-3P	β (1–6); β (2–6); β (15–24); β (15–24)-1P; β (15–24)-2P; β (15–24)-3P; β (15–24)-4P; β (7–14)
β-CN (1–25)+Na_2WO_4	β (1–6); β (2–6); β (7–14); β (7–24); β (7–24)-1P; β (2–25); β (2–25)-2P	β (1–6); β (2–6); β (7–14); β (15–24); β (15–24)-1P; β (1–25); β (2–25); β (2–25)-1P
Fe–β-CN (1–25)	β (1–6); β (2–6); β (1–24); β (7–14); β (14–24); β (14–24)-1P	β (2–6); β (7–14); β (14–24); β (14–24)-1P
Fe–β-CN (1–25)+Na_2WO_4	β (1–6); β (2–6); β (1–24); β (7–14); β (14–24); β (14–24)-1P	β (2–6); β (7–14); β (14–24); β (14–24)-1P

β-CN (1–25) was either free or complexed with iron.
The enzymatic reactions were monitored by HPLC coupled to ion spray mass spectrometry.

in perfusion solute (t) and in the effluent (e), respectively. D and T are the delivery rate (ml/min) and the time of collection, respectively.

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

$$Q2 = ([Fe]m - [Fe]m0) \times Pm$$

where m is the segment of perfused intestinal mucosa and Pm 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 h with a Fe-free solute.

Net Fe disappearance (Fe abs, μmol) during the perfusion was Fe abs=Q1−Q2.

2.4. Statistical analysis

Results of iron disappearance are expressed as mean and standard deviation. Groups were compared by two-way analysis of variance and Student's t test. Differences were considered significant at a probability level of $P<.05$. Data analyses were performed using the StatView SE+Graphics statistical program.

3. Results

Table 1 displays the effect of the inhibition of alkaline phosphatase by Na_2WO_4 on the proteolysis and on the dephosphorylation of β-CN (1–25) either in a free form or complexed to iron. Dephosphorylation of β-CN (1–25) was expressed as the remaining degree of phosphorylation from 4 (native peptide) to 0 (native peptide minus four phosphate groups). The main reaction was the dephosphorylation of the free peptide; it occurred during the first hour of perfusion. After 2 h of perfusion, only small fragments derived from β-CN (1–25) were detected. In the presence of Na_2WO_4, CPP lost only one or two phosphate groups. During perfusion of Fe–β-CN (1–25) complex, only a few small peptides were detected whatever the presence of Na_2WO_4.

Table 2 shows the inhibition of alkaline phosphatase by Na_2WO_4 on iron disappearance. The disappearance of β-CN (1–25)-bound Fe was greater than Fe gluconate; Na_2WO_4 did not change the absorption rate of Fe gluconate; it enhanced the absorption of β-CN (1–25)-bound Fe.

Table 2
Iron absorption (%); effect of the inhibition of alkaline phosphatase by Na_2WO_4

	Control	Na_2WO_4	P
β-CPP Fe	10.6±2.0	16.0±2.8	<.01
Fe Gluconate	8.2±2.5	8.8±4.0	.39
P	.05	<.01	

x±S.D. (n).
ANOVA: form of iron: $F=14.5$; $P=.001$; inhibition of alkaline phosphatase: $F=5.7$; $P=.03$; form of iron×inhibition of alkaline phosphatase: $F=29.9$; $P=.07$ (NS).

4. Discussion

Caseinophosphopeptides can form soluble organophosphate salts and may function as carriers for minerals.

Previous experimental and human studies clearly show that binding iron to the CPP produced by the hydrolysis of β casein improves its bioavailability [9,15]; furthermore, it decreases the susceptibility of peptide sequences to proteases, which could influence their functional and nutritional properties. So the present study focused on the membrane phase of CPP-bound Fe, by assessing the susceptibility of CPP to alkaline phosphatase.

The extensive proteolysis of free CPP which occurred in the present study agrees with results from previous experimental [6,16] and human [17] reports which show a high susceptibility of free CPP to digestive enzymes.

The presence of Na_2WO_4, only slowed the dephosphorylation process, as free CPP still lost one or two phosphate groups, confirming previous reports [12].

The mass spectrometry analysis suggested the following sequence for peptide degradation: (a) hydrolysis of the N-terminal part, (b) dephosphorylation and (c) hydrolysis of the core and the C-terminal region of the molecule.

On the other hand, the Fe–β-CN (1–25) complex was poorly susceptible to both proteolysis and dephosphorylation, and yield only a few small peptides during digestion: iron binding lessened the phosphatase activity to the same level as Na_2WO_4 did. This decreased susceptibility of complexed CPP to brush border enzymes is in line with its previously reported resistance to digestive enzymes in vitro [4] and ex vivo [7]. The crossover between phosphopeptide chains induced by the linkage of iron could explain the observed resistance to digestive enzymes [4,5].

In contrast to our previous finding [7], the phosphorylated region of the peptide, i.e., β-CN (14–24), was detected in the lumen of Fe–β-CN (1–25) perfused rats. This discrepancy is attributed to the higher amount of phosphopeptide used here (14 vs. 3 mg in the previous study).

Native CPP was not detected anymore at the end of this experiment; owing to the resistance of the complex to enzymes, it seems unlikely that all the amount of Fe–β-CN (1–25) perfused was hydrolysed; this observation fits better with an uptake of iron in a bound form, such as suggested in recent studies [9,10]. Other minerals bound to CPP, such as calcium, are better absorbed than in a free form [18]. Alternatively, iron could be released at the close contact of the brush border membrane.

This study gives some insight into the mechanisms of absorption of peptide-bound Fe; the disappearance of β-CN (1–25)-bound Fe was enhanced by the inhibition of alkaline phosphatase activity, which appears therefore as a limiting step. The role of alkaline phosphatase seems to depend on the type of CPP: this study used a pure caseinophosphopeptide from β casein, while conflicting results were obtained with a mixture of CPP issued from the hydrolysis of whole caseins [12]; the hydrolysis of caseins yields several different CPP which differ in amino acid composition,

weight, charge and hydrophobicity [13,19]; so, pure β-CN (1–25) enhances Fe uptake, which seems to be decreased by α s-casein-derived CPP [13]. Releasing Fe from these inhibiting CPP should therefore improve iron absorption, as previously shown [12].

5. Conclusions

This in vivo study assessed the membrane phase of CPP and CPP-bound Fe disappearance. Binding Fe to β-CN (1–25) prevented CPP from dephosphorylation and proteolysis, confirming the low susceptibility of the complex to digestive enzymes.

Inhibiting alkaline phosphatase activity enhanced Fe–β-CN (1–25) disappearance, suggesting that this form of Fe is at least partly uptaken in a bound form. The functional interactions between Fe and CPP seem to be peptide dependent. This effect is specific for every CPP; when bound Fe is not released in vivo and is, at least partly, absorbed in a bound form. That could explain its good bioavailability and its low susceptibility to digestive interactions. β-CN (1–25)-bound Fe could be a good candidate for food fortification.

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