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Influence of Various Phosphopeptides of Caseins on Iron Absorption

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The influence of the origin and kind of caseinophosphopeptide (CPP) on iron absorption was assessed by comparing a commercially available CPP mixture (CPPs) and derived chromatographic fractions with the purified, chemically phosphopeptide of β-casein [β-CN(1−25)] using a perfused rat duodenal loop system; gluconate iron was used as control. Only iron complexed to β-CN(1−25) displayed a better bioavailability than gluconate iron. The results obtained with various chromatographic fractions indicated that phosphopeptides of different origins (α_s- versus β-caseins) display specific effects. These findings contribute to the explanation of the discrepancy about the role of caseinophosphopeptides on mineral bioavailability in vivo.

KEYWORDS: Caseinophosphopeptides; iron absorption

INTRODUCTION

Digestion of milk proteins releases numerous bioactive peptides encoded in the native proteins, which display specific physiological functions on gastrointestinal, immunological, hormonal, and nutritional responses (*1*, *2*). Among them, phosphopeptides (CPPs) issued from casein hydrolysis have the ability of binding and keeping soluble cations (*3*, *4*). Indeed, intact bovine milk proteins keep iron soluble in the digestive tract, but they are inhibitors of its absorption, unless they are hydrolyzed (*5*). Substituting low molecular weight CPPs for native proteins could improve metal absorption.

Both α_s- and β-caseins release CPPs during enzyme hydrolysis. The N-terminal CPP issued from the trypsin hydrolysis of β-casein [β-CN(1−25)] is one of the main CPPs produced in vivo during digestion (*6*). Previous experimental studies showed that β-CN(1−25)-bound iron remains soluble in the digestive tract, where it escapes further enzyme digestion (*7*). The absorption of β-CN1−25-bound iron occurs partly by endocytosis and is more efficient than that of gluconate iron (*8*); repletion with β-CN(1−25)-bound iron results also in a significantly better improvement of hemoglobin and iron tissue concentrations in iron-deficient rats than with $FeSO_4$ (*9*).

This study was designed to assess whether this effect is common to every CPP or if it depends on the kind and origin of the peptides. Therefore, it compared the absorption of iron bound to peptides from β-casein [β-CN(1−25) or nonpurified hydrolysate of β casein] or to a blend of CPPs produced by the hydrolysis of whole cow's milk caseins. To characterize the differential effects of phosphopeptides on iron absorption, the fractions obtained by anion-exchange chromatography of the whole CPPs were further tested.

The experimental model was the perfused duodenal rat loop; in this model iron absorption is controlled by iron status and nutrient−nutrient interactions (*8*).

MATERIALS AND METHODS

Apparatus and Biological Materials. *(1) Production of Caseinophosphopeptides and Fe−Caseinophosphopeptide Complexes. (a) β-Casein Hydrolysate and β-CN(1−25).* Tryptic hydrolysates of β-casein (whole hydrolysate) as well as pure β-casein phosphopeptide [β-CN(1−25)] (purity > 85%) were obtained as previously described (*10*). The proportion of β-CN(1−25) in whole β-casein hydrolysate was 12% (w/w).

(b) Whole CPP. The blend of sodium−caseinophosphopeptides mixture (CCP−Na) was obtained from MD Food (Aarhus, Denmark) as spray-dried powder. They were prepared by enzymatic hydrolysis of whole caseins followed by purification and concentration steps. CPPs−Na contained 0.76 g of protein, 0.09 g of sodium, and 0.03 g of phosphorus per gram of powder.

(c) Fractionation of CPPs. Whole CPPs−Na were fractionated by preparative anion-exchange chromatography using a Pharmacia BioPilot system fitted with a semipreparative column (Source 15Q, 26 × 95 mm, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.02 M Tris-HCl buffer, pH 8. Elution was performed by stepwise gradient with the same buffer containing 0.5 M NaCl, and peptides were detected by absorbance at 214 nm. The collected fractions (F1−F4) were concentrated by ultrafiltration on a 3 kDa membrane and extensively diafiltered with Milli-Q water before lyophilization.

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(d) Characterization of the Fractions. The peptidic content of each fraction was further characterized by electrospray mass spectrometry (ESI-MS) working on-line with a high-performance liquid chromatography (HPLC) system (Waters 625 LC system; Waters, Milford, MA). Chromatographic separation of the phosphopeptides was performed at pH 2 using solvent A (0.1% trifluoroacetic acid and 5% acetonitrile in water) and solvent B (0.1% trifluoroacetic acid and 80% acetonitrile in water). Samples, adjusted to pH 2 with 5% trifluoroacetic acid, were introduced into the mass spectrometer (PE-Sciex API III; Sciex, Thornhill, ON, 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 5−80% of solvent B in solvent A over 45 min. The ion source voltage of the mass spectrometer was set at 4.8 kV, and the orifice voltage was set at 80 V. The quadrupole mass analyzer was scanned over a mass/charge (m/z) range of 500−2400 Da with a step size of 0.3 Da. The molecular masses were determined from these data using the software package supplied by Sciex (Biomultiview 1.2). The relative proportion of the major phosphopeptides in each fraction was estimated from their contribution to total ionization intensity.

(2) Iron Binding to CPP. Binding of iron to hydrolyzed β-casein, β-CN(1−25), and CPPs or their purified fractions was performed by mixing them with $FeCl_2$ solution (Fe/phosphopeptides molar ratio = 4) for 1 h at 37 °C, pH 6.5. The resulting solutions were then ultrafiltered and diafiltered on a 3 kDa membrane to remove free minerals. The amount of Fe complexed to phosphopeptides and the presence of traces of calcium and sodium were determined by atomic absorption spectrometry (Varian; model AA 1275) on freeze-dried samples. The prepared complexes contained 0.07 g of iron per gram of phosphopeptides or per gram of whole hydrolysate with 0.1 mg of calcium/g and <1 mg of sodium.

(3) Apparatus of the Intestine Perfusion Experiment. Female adult Sprague−Dawley rats weighing 240−300 g were studied after an overnight fast as previously described (*8*). Animals were anesthetised with sodium pentobarbital; the duodenum was perfused at a delivery rate of 0.16 mL/min; every element of the perfusion device was previously washed with a solution of Triton X-100 (1 g/L) to prevent any contamination. The perfusion solute was kept at 37 °C by thermostatic control, and a nonabsorbable marker (polyethylene glycol 4000) was added to assess actual net water fluxes. After 2 h of perfusion, the animal was killed by an overdose of Doléthal; the perfused loop was washed with EDTA solution to remove iron that could be adsorbed to the outside surface of mucosa, withdrawn, and dried.

Fe was measured by atomic spectrometric absorption (Perkin-Elmer 3030) in serum, perfusion solute, digestive effluent, and the perfused segment of duodenum; tissue was digested in nitric acid at ambient temperature during 24 h. Ringer−Lavoisier solute was used as blank.

Polyethylene glycol (PEG) was measured in the perfusion solute and the digestive effluent by a turbidimetric method described by Hydén (*11*).

Fe disappearance from digestive lumen (Q_1, μmol) was calculated as

$$Q_1 = (1 - [\mathrm{PEG}]_t/[\mathrm{PEG}]_e)([\mathrm{Fe}]_e/[\mathrm{Fe}]_t)DT[\mathrm{Fe}]_t$$

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

Fe stored by the mucosa (Q_2, μmol) during the perfusion was calculated as

$$Q_2 = ([\mathrm{Fe}]_m - [\mathrm{Fe}]_m{}^0)P_m$$

where m is the segment of perfused intestinal mucosa and P_m its dry weight; $[Fe]_m{}^0$ is Fe concentration of the duodenal mucosa immediately distal to the perfused segment.

Net Fe absorption (Fe abs, μmol) during the perfusion was

$$\text{Fe abs} = Q_1 - Q_2 - \text{EDTA-Fe}$$

where EDTA-Fe is the amount of Fe removed from the mucosa by EDTA washing.

Table 1. Uptake, Mucosal Retention, Net Absorption by the Duodenal Rat Loop of Iron Gluconate, and Iron Bound to the Purified β-CN(1–25), to Nonpurified Hydrolyzed β-Casein, or to a Blend of CPPs Produced by the Digestion of Whole Cow's Milk Caseins[a]

		uptake				
	n	disappearance from lumen	adsorbed Fe[b]	net uptake	mucosal storage	net absorption
CPPs	9	6.5 ± 1.9 a	1.4 ± 0.6 a	5.1 ± 1.5	1.1 ± 1.4	4.0 ± 1.5
β-Cas H	5	8.1 ± 2.3	1.3 ± 0.6 a	6.8 ± 2.0	1.8 ± 0.8	5.0 ± 1.8 a
β-CN(1–25)	14	8.2 ± 2.1 a	1.2 ± 0.2 a	7.0 ± 2.1	1.7 ± 1.2	5.4 ± 1.7 a
gluconate	7	11.9 ± 7.2	7.2 ± 4.6	4.8 ± 5.4	3.0 ± 3.0	1.7 ± 4.8
ANOVA		$p = 0.046$	$p < 0.0001$	$p = 0.256$	$p = 0.185$	$p < 0.036$

[a] Percent of amount perfused; mean ± SD. Gluconate, ferrous iron gluconate; CPPs, CPPs-bound Fe; β-Cas H, Fe bound to whole hydrolyzed β-casein; β-CN(1–25), Fe bound to β-CN(1–25). Values followed by an "a" are not equal to gluconate. [b] Adsorbed Fe, Fe removed by washing the perfused mucosa with EDTA. [c] ANOVA followed by Fisher test ($p < 0.05$).

Protocol of Perfusion Experiments. In a first experiment four groups (n = 5−14/group) of rats were studied. The composition of perfusion solute was adapted from Ringer−Lavoisier solute; its pH was adjusted to proximal duodenum pH (5.5) and contained 100 μmol/L Fe in either form.

Fe was bound either to β-CN(1−25)[β-CN(1−25)/Fe complex], to hydrolyzed β-casein (β-cas H/Fe), to whole CPP (CPPs−Fe), or as iron (Fe^{2+}) gluconate.

In a second set of experiments rats (n = 5−10/ group) were perfused with the four fractions (F1−F4) issued from CPPs chromatographic fractionation.

RESULTS

In the first set of experiments, we compared iron uptake from three different iron complexes [β-CN(1−25)/Fe, β-casein hydrolysate/Fe and CPPs/Fe] and iron gluconate. The net uptake of the different sources of iron did not differ; however, a significant amount of gluconate iron, which had disappeared from the digestive lumen, was in fact adsorbed to the outside face of the apical membrane and was released from the mucosa by EDTA (**Table 1**). Iron bound to β-CN(1−25) or to nonpurified β-casein hydrolysate displayed a nonsignificantly higher uptake and a nonsignificantly lower mucosal retention than iron given as CPPs/Fe complex; as a result, the net absorption of iron bound to these peptides was significantly higher than that of gluconate iron (**Table 1**).

Mass spectrometry analysis showed that the major phosphopeptide sequences contained in whole CPPs preparation were [α_{s1}-CN(43−58), two phosphoserine residues], [α_{s1}-CN(59−79), five phosphoserine residues], [α_{s2}-CN(53−70), four phosphoserine residues], [α_{s2}-CN(46−70), four phosphoserine residues], [α_{s2}-CN(2−21), four phosphoserine residues], [β-CN(33−48), one phosphoserine residue], and [β-CN(1−25), four phosphoserine residues]. The relative proportion of β-CN(1−25) in the mixture was ∼35%. This mixture was fractionated, and the role of each fraction on iron absorption was tested. As shown in **Figure 1**, differences were detected between fractions. Iron bound to CPP of fraction F2 displayed a nonsignificantly higher apical uptake and a nonsignificantly enhanced transfer compared with other fractions. This resulted in a significantly higher net absorption than iron bound to F1 ($p = 0.025$) and F3 ($p = 0.010$); the p value between F2 and F4 was 0.061.

The major peptides detected in each fraction are given in **Table 2**. As expected, fraction 1 contained less negatively charged phosphopeptides, whereas the most negatively charged molecules were eluted in fraction 4. F1 contained a mixture of phosphopeptides derived from the two major phosphorylated

Table 2. Identification and Properties of the Major Phosphopeptides in Four Fractions Obtained by Fractionation of Whole Phosphopeptides of Bovine Caseins on an Anion-Exchange Column at pH 8

fraction	casein	variant	sequence	no. of phosphate groups	charge at pH 8	measured mass	relative proportion[a] (%)
F1	α S1	B	f(106–119)	1	–3.97	1660.7	10
	α S1	B	f(43–58)	2	–8.9	1927.8	20
	α S1	B	f(43–58)[b]	2	–8.9	1943.8	20
	α S1	B	f(104–119)	1	–3	1952.1	15
	b	A1	f(33–48)	1	–7	2062.0	5
	b	A1	f(30–48)	1	–7	2353.1	5
	b	A1	f(1–25)[c]	3	–10.9	3043.0	5
	α S1	B	f(106–132)	1	–3	3149.5	7
F2	α S2	A	f(2–21)	4	–11	2619.4	8
	α S2	A	f(2–21)[d]	4	–11	2635.4	10
	b	A1	f(1–25)	4	–13.9	3124.0	80
F3	α S2	A	f(2–20)	4	–11	2491.2	22
	α S1	B	f(59–79)	5	–13.9	2704.4	45
	α S2	A	f(46–70)	4	–15	3008.6	15
F4	α S1	B	f(59–79)	5	–14.9	2704.365	55
	α S2	A	f(46–70)	4	–15	3008.626	30

[a] Determined from the relative contribution of individual sequences to total ionization intensity. [b] Oxidized form of native fragment f(43–48). [c] Dephosphorylated form of native fragment f(1–25). [d] Oxidized form of native fragment f(2–21).

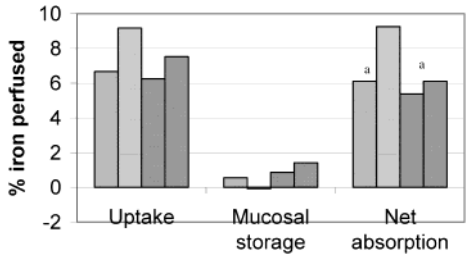

Figure 1. Mean uptake, mucosal storage, and net absorption (percent of amount perfused) of iron bound to fractions issued from chromatography fractionation of whole casein hydrolysate. Initial iron concentration = 100 μmol. F1, $n = 10$; F2, $n = 9$; F3, $n = 8$; F4, $n = 5$. (Within each grouping of bars, bars represent, from left to right, F1, F2, F3, and F4.) Uptake: ANOVA, $p = 0.139$. Mucosal retention: ANOVA, $p = 0.282$. Net absorption: ANOVA, $p = 0.043$. Fisher's test: a ≠ F2 ($p < 0.05$).

caseins, that is, α_{s1}- and β-caseins. F3 and F4 contained the most phosphorylated sequences from α_{s1}- and α_{s2}-caseins, whereas the highly phosphorylated peptide from β-casein, that is, β-CN(1−25) was eluted in fraction F2. Some chemical modifications that have occurred during processing, for example, dephosphorylation or methionine oxidation, were also detected. The major peptides detected were fragment f(43−58) from α_{s1}-casein and fragments f(1−25) and f(2−25) from β-casein (**Table 2**).

DISCUSSION

Digestion of caseins releases several CPPs with functional properties; they display clusters of phosphoserine that bind and keep minerals soluble (*3*). Some studies have shown that these molecules enhance the absorption and bioavailability of calcium and other minerals, that is, zinc and iron (*6*, *9*, *12*−*14*), whereas other studies have failed to show any positive effect (*14*−*16*). This discrepancy might have originated from differences in experimental design, mineral status of the animals, or physicochemical composition of CPPs blend used as mineral carrier. Indeed, distinction must be clearly made between studies in which CPPs were used in free, noncomplexed form, and those in which CPPs/mineral complexes were pre-performed before animal studies.

The present work confirms our previous conclusions about the positive role of β-CN(1−25) on iron absorption in nondeficient rats. Indeed, in agreement with recent results, iron absorption is improved only by the Fe−CPPs complex and not when free peptides are added to Fe (*9*, *17*).

Moreover, our results underscore the importance of the physicochemical properties of CPP toward iron absorption. The efficiency of various CPPs on iron absorption depends on their origin and their structural properties. Iron bound to the phosphopeptide produced by digestion of β-casein displayed a better absorption than whole CPPs; the fractions rich in β-casein-derived phosphopeptides (F1 and F2) displayed a better iron absorption than α_s-caseins fractions (F3 and F4, **Figure 1**); the best iron absorption level was provided by fraction F2, which is the only one that contained phosphorylated β-CN(1−25).

Only some assumptions can be raised on the mechanisms involved in the differences observed in the metabolism of iron complexed to different CPPs. They concern the relative affinity of individual phosphopeptide sequences toward iron. It is generally believed that the clusters of phosphoserine residues are the determining factor for mineral binding. However, binding of ferrous iron to CPP is different from that of other divalent cations such as calcium; it is characterized by higher affinity, oxidation into ferric iron, and high stability to pH variation (*18*, *19*). These physicochemical properties are probably different from one phosphopeptide sequence to another. Although structural data are lacking on CPPs, the assumption above is supported by the results of Gaucheron et al. (*20*), who showed that binding of iron induced structural changes that differed between αs- and β-caseins. Consequently, we suggest that for a given phosphopeptide, there is a correlation between mineral complexation and the accessibility of iron−peptide complexes to apical membrane and transport systems. The affinity constant must be optimal for a positive biological effect. In this sense, the β-CN(1−25) sequence, with four phosphoseryl and seven glutamic acid residues, exhibits such properties. F1 CPP could not display enough sites for an efficient iron binding, whereas iron could be too tightly bound to the highly electronegative phosphoryl clusters of fractions F3 and F4 (*7*, *17*). To check these assumptions, experiments are in progress to determine the relative affinity constants, the chelating capacity, and the stability of various phosphopeptide−iron complexes toward brush border membrane enzymes.

Whatever the mechanisms involved in the differences of iron absorption between the different fractions, these results suggest that the differences in iron absorption between cow's milk and human milk could be partly explained by their differences in protein composition, because human milk's caseins do not contains α-casein (*21*).

ABBREVIATIONS USED

CPP, caseinophosphopeptide; CPPs, mixture of CPP produced by whole casein hydrolysis; β-CN(1−25) = 1−25 CPP of β-casein.

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