Original article

Improvement of zinc intestinal absorption and reduction of zinc/iron interaction using metal bound to the caseinophosphopeptide 1-25 of β-casein

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Abstract – Binding zinc (Zn) to soluble caseinophosphopeptides (CN), produced by the hydrolysis of caseins, improves its absorption and could prevent inhibition by other nutrients such as iron (Fe). The absorption of Zn (100 µmol/L) bound to the 1-25 CN (β-CN(1-25)) of β-casein, or as $ZnSO_4$ was studied using the isolated, perfused rat intestinal loop system. Fe (Fe–CN or Fe gluconate (Fe Gluc)) was added at Zn/Fe ratios of 2:1, 1:5 and 1:10. Disappearance from the lumen (Q1) and net absorption (ZnAbs) of Zn–CN were statistically greater than for $ZnSO_4$; Zn retention by the mucosa (Q2) did not significantly differ. Fe Gluc reduced Q1, Q2 and ZnAbs for $ZnSO_4$ at ratios of 1:5 and 1:10 and for Zn–CN at a ratio of 1:10. Fe–CN reduced Q1 and ZnAbs of both forms of Zn at a ratio of 1:10; Q2 remained unchanged. Binding Zn to β-CN(1-25) improved Zn absorption and prevented Fe from inhibiting its absorption. © Inra/Elsevier, Paris

zinc / iron / digestive absorption / caseinophosphopeptide / rat

Résumé – Augmentation de l'absorption intestinale du zinc et diminution des interactions zinc/fer en utilisant un métal lié au caséinophosphopeptide 1-25 de la β-caséine. La fixation du zinc (Zn) à des caséinophosphopeptides solubles (CN) produits par l'hydrolyse enzymatique des caséines pourrait augmenter son absorption et limiter l'inhibition exercée par d'autres nutriments tel le fer (Fe). L'absorption du Zn sous forme liée au 1-25 CN (β-CN(1-25)) de la β-caséine ou de $ZnSO_4$ a été étudiée par la méthode de l'intestin de rat isolé, perfusé. Le Fe (Fe–CN ou Gluconate (Fe Gluc)) a été ajouté à des rapports Zn/Fe de 2:1, 1:5 et 1:10 Zn/Fe. La disparition du Zn–CN de la lumière intestinale (Q1) et son absorption nette (ZnAbs) étaient significativement plus fortes que celles du $ZnSO_4$; la rétention muqueuse du Zn (Q2) ne diffère pas significativement entre les deux

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séries. Fe Gluc a diminué Q1, Q2 et ZnAbs de $ZnSO_4$ aux rapports 1:5 et 1:10, ne diminuant que Q1 et ZnAbs pour Zn–CN au rapport 1:10. Fe–CN a diminué Q1 et ZnAbs, au rapport 1:10 pour les deux formes de Zn. La fixation du Zn au β-CN (1-25) augmente son absorption et assure sa protection contre l'interaction inhibitrice du Fe. Les mécanismes en cause pourraient impliquer une augmentation de la solubilité intraluminale du Zn et l'étape de transfert entérocytaire. © Inra/Elsevier, Paris

zinc / fer / absorption digestive / caséinophosphopeptide / rat

1. INTRODUCTION

Zinc (Zn) is the cofactor of several hundred metalloenzymes involved in protein and nucleic acid synthesis [27]. Zn deficiency leads to anorexia [30], and affects tissues capable of rapid proliferation. A lack of Zn during critical prenatal and postnatal periods therefore impairs growth, bone and cerebral development [26, 30], gastro-intestinal tract function and immunity. However, Zn deficiency can also be an isolated cause of reduced growth rate [32].

Zn is mainly absorbed in the jejunum [1, 18]. Absorption depends on the dietary form of the Zn and on the presence of certain intraluminal factors. Thus, lactose and citric acid [23, 29] facilitate Zn uptake by the enterocyte, whereas phytates decrease Zn absorption [22]. Iron (Fe) inhibits Zn uptake and metabolism and Zn has a similar effect on Fe absorption [3, 11, 13, 17, 31].

Binding Zn to ligands such as dietary proteins can keep it soluble and enhance its intestinal absorption. The hydrolysis of caseins yields caseinophosphopeptides (CN) which bind calcium via their phosphoserine residues [2, 33]. Other divalent cations, such as Zn and Fe, can bind to phosphoserine residues in proportion to their degree of phosphorylation and according to the cation [4, 9, 33]. These CN are normally found in the intestinal lumen during digestion [20, 21]. They are somewhat resistant to digestive enzymes [4, 5, 28]. In vivo studies on the influence of CN on mineral absorption have given conflicting results. CN improve calcium absorption in rats by preventing the precipitation of calcium salts in the small intestine [28, 35] but have no effect in the pig [24]. Other studies have shown them to have a limited effect on calcium absorption in the rat ileum [19]. β-CN (1-25) is produced by the hydrolysis of β-casein, and has four of the five phosphoserine residues of the native protein. Thus 1 mole can bind 4 moles of Fe or Zn with a greater affinity than for calcium [4]. Our previous studies showed that binding Fe to β-CN (1-25) increased its net absorption by normal and Fe deficient rats and decreased the inhibitory effect of calcium on Fe absorption [25]. The addition of CN to a solution containing phytates also increases the absorption of calcium and Zn in rat pups [14] and in adult humans [15].

The present study was performed to confirm in vivo the improved absorption of Zn in the presence of β-CN (1-25) and to assess the influence of β-CN (1-25) on the inhibition of Zn absorption by Fe. The absorption of Zn bound to purified β-CN (1-25) was compared to that of Zn sulfate using the isolated jejunal rat loop model. The inhibition of Zn absorption by Fe was studied using different concentrations of Fe as β-CN (1-25) bound Fe or Fe gluconate.

2. MATERIALS AND METHODS

2.1. Preparation of the 1-25 caseino-phosphopeptide of β-casein (β-CN (1-25))

β casein (β-CN) was isolated from industrially produced sodium caseinate (Armor Protéines, Saint-Brice-en-Coglès, France) by cold solubilization (pH 4.5; 4 °C), followed by ion exchange

chromatography [2, 4]. β-CN (1-25) was obtained by tryptic digestion of β-casein. Fe and Zn were bound to β-CN (1-25) by adding $FeCl_2$ and $ZnCl_2$ to the solution (pH = 5.3; 30 min; 25 °C (Milli Q system, Millipore)). Unbound Fe and Zn were removed by ultracentrifugation and filtration through a regenerated cellulose membrane with a 3 000-Da cut-off (membrane SIOY3, Amicon, Lexington, MY). The resulting complex was freeze-dried. Complexed Fe and Zn and residual calcium were measured by atomic absorption spectrometry (Varian AA 1275). One mole of peptide bound 4 moles of Fe or Zn. A control without β-CN (1-25) was incubated and dialysed under the same conditions, to be sure that the variations of Zn and Fe concentrations were actually induced by binding to the phopshopeptide.

2.2. Animals

Adult female Sprague-Dawley rats weighing 250–300 g were starved for 12 h before study but had free access to water. The rats were assigned to 14 groups (six animals/group): rats were perfused with 100 µmol/L Zn sulfate ($ZnSO_4$), or Zn bound to β-CN (1-25) (Zn–CN) alone or in the presence of Fe gluconate (Fe Gluc) (which remained soluble above pH = 4), or Fe bound to β-CN (1-25) (Fe–CN). The Fe concentrations were 50 µmol/L, 500 µmol/L, 1 000 µmol/L giving Zn/Fe ratios of 2:1, 1:5 and 1:10. The absence of Zn adsorption to the digestive mucosa, which could have falsely improved the absorption rate, was checked by perfusing a group of dead rats. Another group of rats was perfused with a Zn-free solution to confirm that no significant Zn secretion occurred during the experiment.

2.3. Perfusion

The perfusion solute was adapted from Ringer–Lavoisier solute and was checked to be free of Zn contamination. Its pH was adjusted to that of the proximal jejunum pH (pH = 7); it was isotonic to plasma (285–300 mosmol) and contained 100 µmol/L Zn as sulfate or Zn–CN. Rats were anesthetized with Ketamine (Ketalar™), which has no effect on gut motility. The erythrocytes were counted to prevent from anaemia which could disturb Fe absorption. Then the jejunum was exposed by a laparotomy. The loop was perfused through a catheter inserted at the angle of

Treitz; effluent was collected 5 cm lower. Solid material was washed out with 1 g/L Triton X100 in water to prevent any contamination. The perfusion solute was kept at 37 °C and was delivered at 0.16 mL/min for 2 h using a peristaltic pump to avoid loop distension; a non-absorbable marker (polyethylene glycol 4 000) was added to assess net water fluxes. The rat was killed with an overdose of Doléthal™, the perfused loop was washed with saline, withdrawn and dried in a oven at 90 °C to constant weight.

2.4. Assays

Zn and Fe concentrations in perfusion solute, gut effluent and the mucosa of the perfused segment were measured by atomic absorption (Perkin Elmer 3030); tissue was digested in 10 N nitric acid at ambient temperature for 24 h. Ringer–Lavoisier solute was used as blank.

Polyethyleneglycol (PEG) in the perfusion solute and the gut effluent was measured by a turbidimetric method [16].

The disappearance of Zn from the gut lumen (Q1: µmol) was calculated from:

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

where [PEG] and [Zn] were the PEG and Zn concentrations in the perfusion solute (t) and in the effluent (e). D and T are the delivery rate (mL/min) and the time of collection.

The Zn stored by the mucosa (Q2: µmol) during perfusion was calculated from:

$$Q2 = ([Zn]m - [Zn]m0) * Pm$$

where [Zn]m is the Zn concentration of perfused intestinal mucosa segment and Pm its weight; [Zn]m0 the Zn concentration of intestinal mucosa segment perfused with a Zn-free solute.

Net Zn absorption (Zn abs: µmol) during the perfusion was:

$$Zn\ abs = Q1 - Q2.$$

2.5. Statistics

Results are expressed as means and standard deviations. Groups were compared by two-way ANOVA followed by Student's t-test using 'Statview SE + Graphics™' within each group

(ZnSO$_4$ or Zn–CN). The effect of Fe is expressed as a ratio of the mean for the control Fe-free group (%). These values were also compared by ANOVA and Student's t-test. Significance was set at $P < 0.05$.

3. RESULTS

3.1. Absorption of ZnSO$_4$ and Zn–CN

Fe-free Zn absorptions are given in the first lines of *tables I* and *II*.

No significative change in Zn concentration in the perfusion solute was observed (100.12 µM before and after experience) while perfusing dead rats with ZnSO$_4$: Zn disappearance from gut lumen was only due to Zn uptake by enterocytes and not to Zn adsorption to the digestive mucosa.

Zn–CN was lost from the intestinal lumen (Q1) significantly faster than ZnSO$_4$. Zn

retention by the mucosa (Q2) of the two groups was essentially the same. The net absorption (ZnAbs) of Zn–CN was statistically greater than that of ZnSO$_4$.

3.2. Effect of different forms of Fe on ZnAbs

The influence of different forms of Fe on ZnSO$_4$ and Zn–CN absorption are shown in *tables I* and *II*. Low concentrations of Fe Gluc (Zn/Fe ratio: 2:1) significantly reduced Q1 and ZnAbs of ZnSO$_4$ (*table I*). Higher concentrations of Fe Gluc (Zn/Fe ratio: 1:5; 1:10) inhibited these two parameters and Q2. Inhibition was significantly greater at a Zn/Fe ratio of 1:10.

Fe–CN reduced significantly Q1 and ZnAbs at a Zn/Fe ratio of 1:10. The influence of the form of Fe on Zn absorption

Table I. Effect of iron on zinc sulfate absorption

		% Q1	% Q2	% ZnAbs Anova
1	ZnSO$_4$	27.629 ± 0.003 $P < 0.001$	7.579 ± 0.001	20.050 ± 0.003
2	Fe Gluc (50 µM)	27.383 ± 0.003 $P < 0.001$	7.568 ± 0.001	19.815 ± 0.004
3	Fe Gluc (500 µM)	23.042 ± 0.001[a,b] $P < 0.001$	5.451 ± 0.001[a,b]	17.591 ± 0.002[a,b]
4	Fe Gluc (1000 µM) ANOVA	22.160 ± 0.002[a,b,c] $P < 0.001$	5.195 ± 0.001[a,b,c] $P < 0.001$	16.966 ± 0.002[a,b,c] $P < 0.001$
5	FeCN (50 µM)	27.299 ± 0.001 $P < 0.001$	7.353 ± 0.002	19.946 ± 0.001
6	FeCN (500 µM)	27.374 ± 0.003[*] $P < 0.001$	7.574 ± 0.002[*]	19.820 ± 0.005[*]
7	FeCN (1000 µM) ANOVA	26.978 ± 0.002[a,d,*] $P < 0.001$	7.523 ± 0.003[*] NS	19.455 ± 0.005[a,d,*] $P < 0.05$

Results are expressed as a ratio to the total amount of perfused Zn (%); mean ± 1 SD.
Rats were perfused with perfusion solute containing Zn sulfate (100 µmol/L); Fe gluconate (Fe Gluc) or Fe bound to β-CN (1-25) (Fe–CN) was added at different concentrations. Q1: Zn lost from gut lumen; Q2: Zn stored by the mucosa; ZnAbs: absorbed Zn; [a] Different ($P < 0.001$) from control group ZnSO$_4$ (1); [b] different ($P < 0.001$) from (2); [c] different ($P < 0.001$) from (3); [d] different ($P < 0.001$) from (5); [*] different ($P < 0.001$) from group perfused with a different form of Fe but same concentration of Fe.

was assessed by comparing groups perfused with the same Fe concentrations and the same form of Zn. Q1, Q2 and ZnAbs were significantly different when Fe was present as Fe Gluc and as Fe–CN at Zn/Fe ratios of 1:5 and 1:10.

Table II shows that low concentrations (2:1 ratio) of Fe Gluc did not reduce Q1, Q2 and ZnAbs for Zn–CN. Higher concentrations of Fe Gluc (Zn/Fe ratio: 1:5 and 1:10) reduced Q1 and ZnAbs; this inhibition was significantly greater when the ratio was 1:10.

Fe–CN at the ratio of 1:10 significantly inhibited Q1 and ZnAbs. Perfusion with the same concentrations of Fe Gluc or Fe–CN had significantly different effects on Q1 and ZnAbs when the ratio was 1:10.

Figure 1 shows the results expressed as the inhibition of absorption of the two forms of Zn by the two forms of Fe. This inhibition was significantly greater while perfusing $ZnSO_4$ with low Zn/Fe ratios (1:5 and 1:10), but was similarly weak when Zn (as $ZnSO_4$

or Zn–CN) was perfused with Fe Gluc (at 2:1 ratio) or Fe–CN (at any ratio). This inhibition became statistically lower in rats perfused with Zn–CN than in rats perfused with $ZnSO_4$ at low Zn/Fe ratios (1:5 and 1:10).

4. DISCUSSION

Zn absorption in the jejunum [18] is influenced by Zn status [27], owing to the synthesis of metallothionein, a cysteine-rich protein [8]. But it is passive and linearly proportional to the luminal concentration of Zn between 0.1 mol/L and 1.8 mol/L [18]. Thus Zn absorption is influenced by the form of dietary Zn [31] and by interactions with substances in the gut which enhance absorption, such as proteins and glucose polymers [27, 29], or inhibit it such as phytates or other trace elements (Fe) which compete for common carriers [7].

Athough caseinophosphopeptides (CN) positively influence Fe absorption [25], they

Table II. Effect of iron on absorption of zinc bound to β-CN (1-25)

		% Q1	% Q2	% ZnAbs
8	Zn–CN	37.395 ± 0.008	7.365 ± 0.002	30.030 ± 0.008
9	Fe Gluc (50 μM)	37.041 ± 0.001	7.339 ± 0.001	29.702 ± 0.001
10	Fe Gluc (500 μM)	36.263 ± 0.002[a,b]	7.352 ± 0.004	28.911 ± 0.003[a,b]
11	Fe Gluc (1000 μM)	35.180 ± 0.002[a,b,c]	7.134 ± 0.003	28.046 ± 0.004 [a,b,c]
	ANOVA	$P < 0.001$	NS	$P < 0.001$
12	Fe–CN (50 μM)	37.041 ± 0.004	7.277 ± 0.002	29.764 ± 0.005
13	Fe–CN (500 μM)	36.618 ± 0.003	7.310 ± 0.004	29.308 ± 0.005
14	Fe–CN (1 000 μM)	36.161 ± 0.005[a,d*]	7.277 ± 0.003	28.885 ± 0.007[a,d*]
	ANOVA	$P < 0.001$	NS	$P < 0.05$

Results are expressed as a ratio to the total amount of perfused Zn (%); mean ± 1 SD.
Rats were perfused with perfusion solute containing Zn bound to β-CN (1-25) (100 μmol/L). Iron was added as Fe gluconate (Fe Gluc) or bound to β-CN (1-25) (Fe–CN). Q1: Zn lost from gut lumen; Q2: Zn stored by the mucosa; ZnAbs: absorbed Zn; [a] Different ($P < 0.001$) from control group Zn–CN (8); [b] different ($P < 0.001$) from (9); [c] different ($P < 0.001$) from (10); [d] different ($P < 0.001$) from (12); [*] different ($P < 0.001$) from group perfused with a different form of Fe but with same concentration of Fe.
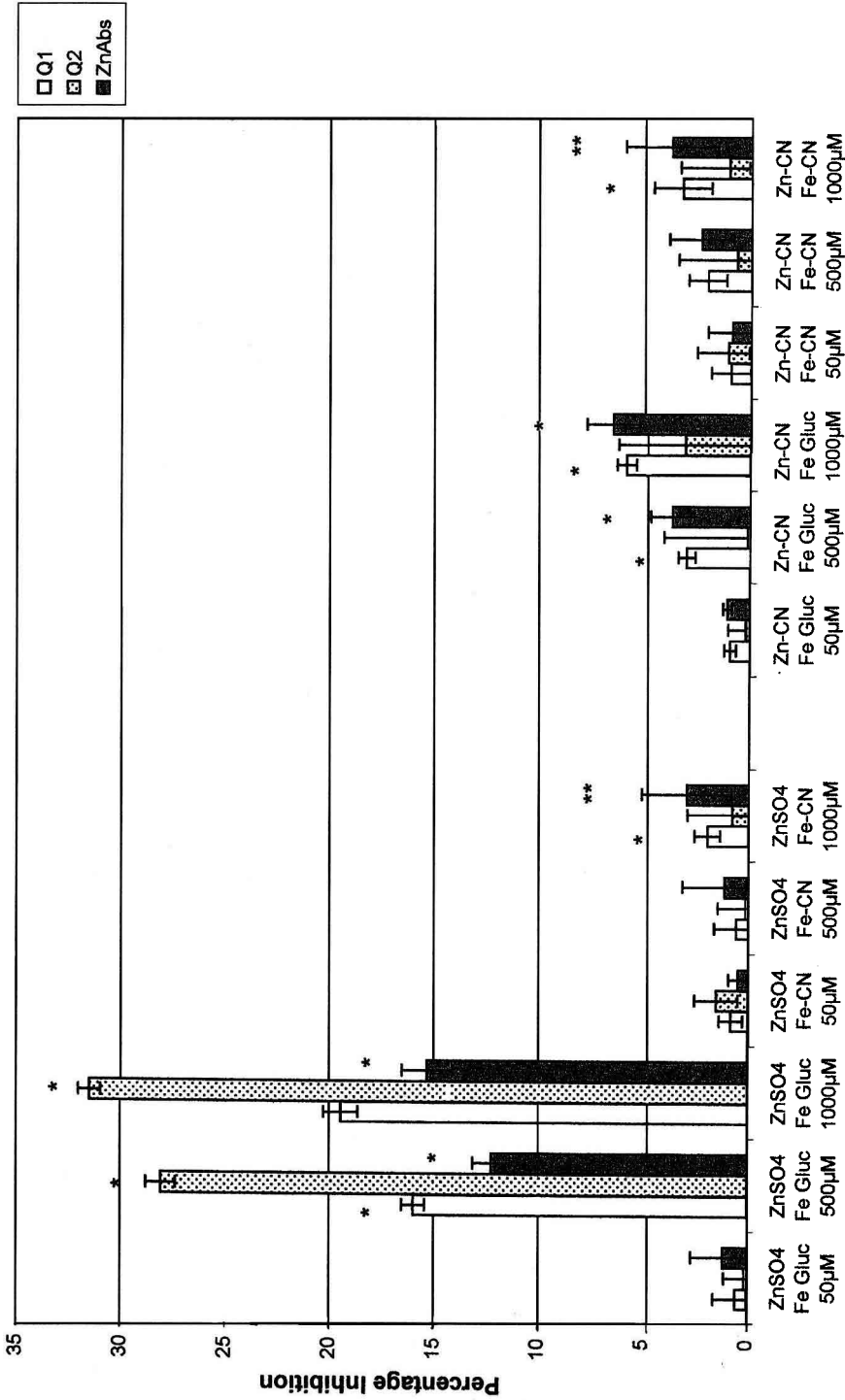
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Figure 1. Inhibition of zinc absorption by different forms of iron. Results are expressed as the ratio to the mean value of the iron free group (%). 100 µmol/L Zinc (ZnSO$_4$ or Zn–CN) was perfused in the presence of 50, 500, 1 000 µmol/L iron (Fe Gluc or Fe–CN). Q1: Zn lost from the gut lumen; Q2: Zn stored by the mucosa; ZnAbs: absorbed Zn. Significance of the difference versus control: * $P < 0.001$; ** $P < 0.05$.

have given conflicting results on Zn absorption [14, 15]. The present in vivo study confirms that Zn bound to β-CN(1-25) is absorbed better than the widely used inorganic salt $ZnSO_4$. This effect appears to be greater than previously reported [14]. We used a well-defined purified CN from β-casein (β-CN (1-25)) to which Zn was bound and not just added [14]. The adult rats used in our work also differ from the rat pups and humans [14, 15].

Our results also support our previous findings on the protection against inhibition offered by binding trace elements to β-CN (1-25) [25]. This occurs when one of the trace elements is provided in a bound form, and is enhanced when both are bound. Only the higher Zn/Fe ratio (2:1) showed no inhibition of Zn absorption by Fe in whatever form. Zn absorption can be inhibited by Fe with Zn/Fe ratios below one-half in dietary or fortification forms of trace elements [10, 29], as shown in the present study. However, although Zn absorption was inhibited by Fe Gluc for both forms of Zn at a ratio of 1:5, the absorption of bound Zn remained 60 % higher than that of $ZnSO_4$; Zn absorption was not inhibited by Fe when both trace elements were provided in a bound form at this ratio.

This enhanced Zn absorption could be due to the Zn bound to soluble β-CN (1-25) being protected from insolubilization during digestion [2], or from interactions with other food in the gut, such as phytates [14]. β-CN (1-25) could also influence Zn absorption by the enterocytes, particularly during apical membrane uptake, where inhibitory interactions between trace elements can occur [34]. Fe and Zn could compete for a common metal-ion transporter [12] and carrier proteins involved in trace element transport from membrane receptor into the enterocyte [7]. Changes in the storage of Zn by enterocytes according to the form of perfused Fe support this assumption. Zn binding to β-CN (1-25) could be resistant to hydrolysis at low pH and by digestive enzymes,

but this remains to be demonstrated; Zn could be absorbed by some mechanism other than passive or carrier-mediated uptake. Zn and Fe bound to β-CN (1-25) could be taken up by endocytosis while chelated to a peptide or an amino acid, as reported for dietary peptides and proteins [6].

CONCLUSION

Zn bound to β-CN (1-25) is absorbed better than Zn sulfate. Its absorption is protected from inhibition by Fe. The mechanisms involved remain to be identified. Further studies are needed to validate these results in man, before this bound form of Zn could be proposed as a food additive.

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