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Effect of Zinc on D-Galactose and L-Phenylalanine Uptake in Rat Intestine *in vitro*

A. Lugea[1], A. Barber and F. Ponz

Departamento de Fisiología y Nutrición
Universidad de Navarra, 31008 Pamplona (Spain)

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The effect of zinc on galactose and phenylalanine uptake was studied using rat everted jejunal rings. The rings were incubated for 2 min in oxygenated Krebs-Ringer-Tris (KRT) solution. Galactose and phenylalanine uptake was reduced by zinc in a dose-dependent manner, but not in a time-dependent way. One mM Zn^{2+} but not 0.5 mM Zn^{2+} inhibited galactose transport without modifying sugar diffusion. Na^{+}-dependent phenylalanine transport was reduced by 0.5 mM and 1 mM Zn^{2+}. However, the metal did not change phenylalanine diffusion obtained in the presence of 40 mM L-methionine or Na^{+}-independent phenylalanine transport. Therefore, zinc seems to interact only with the sodium-galactose or sodium-phenylalanine cotransporters. Zinc inhibited sugar and amino acid transport in a non-competitive way, without a significant change in the affinity of the transporters for their substrates and with a Vmax decrease. The inhibitory effect of Zn^{2+} on galactose and phenylalanine uptake was reversed by washing intestinal rings for 5 min with KRT solution. These results suggest that zinc might exert its inhibitory action by a weak binding to chemical groups related with sodium-substrate cotransporters and located in the luminal membrane of the enterocytes.

Key words: Zn, Inhibition, Intestinal transport, Sugar, Phenylalanine.

Zinc is an essential element that interacts with biomembranes and intracellular components and its metabolism is related to many metalloenzymes. Dietary zinc is mainly absorbed by the small intestine and the mechanism involves both passive diffusion and carrier-mediated components (9, 26). Enterocyte zinc is associated with metallothioneins and a newly identified cysteine-rich intestinal protein, exclusively through thiolate bonds to cysteine

Correspondence to A. Barber (Tel. 105600; Fax 105649).

[1]Present address: Unidad de Investigación del Sistema Digestivo, Hospital General Vall d'Hebrón, Universidad Autónoma de Barcelona, Barcelona 08035 (Spain).

residues (4, 17). These proteins may function as intracellular zinc carriers during transepithelial transport and saturate at high luminal zinc concentrations. Zinc also may be bound to proteins of the brush border membrane, some of them related to zinc transport (26), or interacts with other membrane-proteins or enzymes that do need zinc for their activity (1). Thus, zinc modify the activity of Na^+/K^+ ATPase in the renal epithelium (12) and inhibits net absorption of sodium and water in human jejunum (23). Inhibition by zinc of intestinal glucose absorption has been reported in rats (10, 15) and in pig intestine *in vitro* (30). This inhibition was attributed to a direct interaction with the Na^+-glucose cotransporter, since glucose binding site of the transporter has probably several -SH groups. Similarly, zinc inhibits the transport of galactose and threonine in the rabbit jejunum *in vivo* (21, 22). JAEGER (11) demonstrated that zinc and cadmium absorption are related to glucose and sodium absorption but he speculated an effect at the basolateral membrane level.

The inhibition of galactose and phenylalanine uptake in rat jejunum *in vitro*, and *in vivo* by cadmium and copper (13, 20), metals chemically related to zinc have previously been studied. Cadmium may inhibit zinc activities at many stages interfering with zinc absorption, distribution to different tissues and transport into cells or into several intracellular structures (6). Therefore, it is reasonable to assume that zinc may induce changes on intestinal transport similar to cadmium. Results showed that those heavy metals inhibited the Na^+-dependent galactose and phenylalanine transport in a non-competitive way whereas they did not modify the passive uptake. In the present study, the effect of $ZnCl_2$ on galactose and phenylalanine transport in rat jejunum is investigated and kinetically characterized *in vitro*.

Materials and Methods

Animals.– Albino Wistar rats of either sex weighing 150-250 g were used. The animals were housed at C.I.F.A. (Centro de Investigación Farmacológica Aplicada, Universidad de Navarra, Spain) under controlled conditions of temperature, humidity and lighting (from 7 am to 7 pm) and provided free access to water and food *ad libitum*. Animals were fasted 24 hours prior to the experiments.

Experimental procedure.– The influx of galactose and phenylalanine into rings of everted rat jejunum was determined according to the tissue accumulation method (3). After 24 hours fast, rats were anesthetized with urethane (1.25 g/kg s.c., Sigma) and the abdomen was incised. A 20 cm length of proximal jejunum was rapidly removed and rinsed gently with cold physiological solution (see below) and everted. Rings of about 0.5 cm in length and 25-30 mg in weigth were cut. Every three rings were incubated for 2 or 5 min at 37 °C with shaking in a oxygenated physiological solution. This solution was Krebs-Ringer-Tris (KRT) containing 140 mM NaCl, 5.6 mM KCl, 3 mM $CaCl_2$, 1.4 mM $MgSO_4.7\ H_2O$, 6.1 mM Tris and 4.9 mM HCl, pH 7.4. All these reagents were of analytical grade (Merck). D-galactose or L-phenylalanine (Sigma) and radioactive tracers D-(1-^{14}C) galactose (61 mCi/mmol, Du Pont) and L-(U-^{14}C) phenylalanine (504 mCi/mmol, Du Pont) were added to the incubation medium up to the final concentration. Zn^{2+} was added to the medium as $ZnCl_2$ (Merck). When a Na^+-free KRT solution was required, Tris osmotically substituted for Na^+.

Samples were taken from the incubation medium initially and after the period of incubation for radioactive counting. At the end of the incubation period rings were rapidly removed, rinsed in cold KRT, weighed and kept overnight in 0.5 ml 0.1 HNO_3 at 4 °C. Aliquot samples were taken for liquid scintillation counting (LS 1800, Beckman). The uptake of D-galactose and L-phenylalanine were estimated from the relation between the cpm of the incubation medium samples and the cpm of the HNO_3 aliquots. The substrate uptake were expressed as nmoles of substrate/100 mg wet weight (w.w.). In some experiments, 0.5 mM phlorizin (Sigma) or 40 mM L-methionine (Sigma) were added to the incubation medium to block respectively galactose and phenylalanine transport and estimate intestinal diffusion.

A second protocol determined the reversibility of the inhibitory effect of Zn^{2+} on galactose and phenylalanine uptake. The intestinal rings were preincubated for 2 min in KRT solution containing Zn^{2+} only. After that, the rings were washed for 5 min in KRT solution in the same conditions as the incubation period. Finally, the rings were incubated for 2 min in the physiological solution with galactose or phenylalanine and without Zn^{2+}.

Statistical analysis.– Results of substrate uptake are presented as means + SEM. Significance of differences between groups were analysed by use of the Analysis of Variance for multiple comparisons and the unpaired Student's *t* test for single comparisons. Linear regression analysis was used for the estimation of the kinetic parameters.

Results

Influence of zinc on galactose and phenylalanine uptake.– The effect of $ZnCl_2$ on galactose and phenylalanine uptake in everted rings of rat jejunum has been studied. Galactose uptake by the tissue represents the sum of a diffusion component and a phlorizin-sensitive transport component. Phenylalanine uptake includes three components: passive diffusional uptake, Na^+-dependent transport and Na^+-independent transport.

Figure 1 shows the effect of several zinc concentrations on 0.5 mM galactose uptake. Exposure of the rings to 1 mM Zn^{2+} significantly reduced the galactose uptake (25 % inhibition). The percentage inhibition on galactose uptake was increased as the concentration of the metal increased, but not with the time of zinc exposure. The lowest zinc concentration used (0.5 mM) did not modify galactose uptake. Figure 2 shows zinc inhibition of galactose uptake as a function of galactose concentration (0.5 mM to 20 mM) after 2 minutes of incubation period. One mM Zn^{2+} significantly inhibited sugar uptake

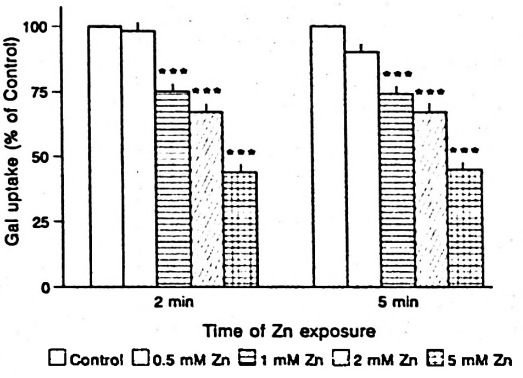

Fig. 1. *Effect of several concentrations of zinc on galactose uptake.*
Intestinal rings were incubated for 2 or 5 min in the presence of 0.5 mM galactose and several concentrations of Zn^{2+} (from 0.5 mM to 5 mM). Values represent percentage of total galactose uptake obtained in the absence of the metal in the incubation medium (Control group). Each value represents the mean + SEM of six intestinal rings per group. *** $p < 0.001$ vs control group.

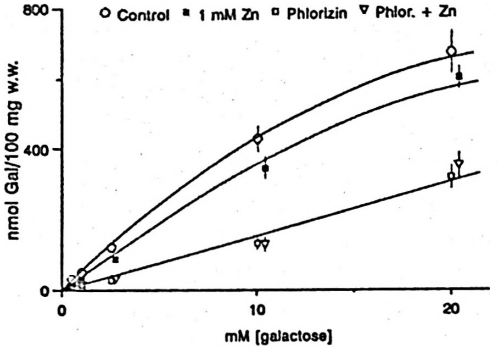

Fig. 2. *Effect of 1 mM zinc on galactose uptake.* Intestinal rings were incubated for 2 min without (total uptake) or with 0.5 mM phlorizin (galactose diffusion) in the presence or absence of 1 mM Zn^{2+} (Control group). Each value represents the mean ± SEM of nine intestinal rings per group.

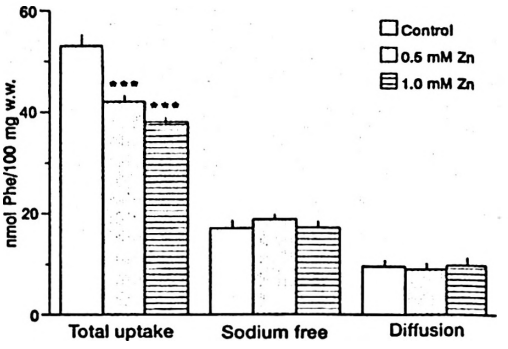

Fig. 3. *Influence of 0.5 mM and 1 mM zinc on phenylalanine uptake.* The inhibition of 0.5 mM phenylalanine uptake by 0.5 mM and 1 mM Zn^{2+} was evaluated in the presence of Na^+ in the incubation medium (total uptake) and in Na^+-free medium. Diffusion was estimated by abolishing total phenylalanine transport with 40 mM L-methionine. Each bar represents the mean ± SEM of nine experimental values. *** $p < 0.001$ vs control group.

at every galactose concentrations used. The metal only reduced galactose transport because it did not change the passive permeability of the tissue evaluated in the presence of 0.5 mM phlorizin.

Phenylalanine uptake was more sensitive to zinc than galactose uptake. At the same concentration of the metal, the inhibition of phenylalanine uptake was greater then that of galactose. As shown in fig. 3, 0.5 mM phenylalanine uptake was significantly reduced by 0.5 mM Zn^{2+} after 2 minutes incubation (20 % of inhibition) and this inhibition was increased by 1 mM Zn^{2+} exposure (30 %). Values of phenylalanine uptake inhibition by 0.5 mM or 1 mM Zn^{2+} measured after 5 min of incubation period were similar to those after 2 min (data not shown). On the other hand, the metal did not change phenylalanine uptake either in the absence of Na^+ in the incubation medium or in the presence of 40 mM L-methionine, indicating that Zn^{2+} only inhibited Na^+-dependent phenylalanine transport (fig. 3). The Na^+-dependent transport values were estimated from the difference between the uptake values obtained in the presence of Na^+ in the incubation medium (total uptake) and in the Na^+-free medium. Na^+-independent transport was obtained from the difference between uptake in Na^+-free medium and the diffusional uptake observed in the presence of 40 mM methionine. The Na^+-dependent transport of 0.5 mM phenylalanine was reduced by 0.5 mM Zn^{2+} (27 %) and by 1 mM Zn^{2+} (43 %) after 2 or 5 min incubation time.

Kinetics of galactose and phenylalanine inhibition by zinc.– The apparent kinetic constants for Na^+-dependent galactose and phenylalanine transport were estimated from Lineaweaver-Burk plot (fig. 4). For galactose transport, an apparent Vmax value of 467.3 nmol/100 mg w.w. and a Km of 10.7 mM were obtained. One mM Zn^{2+} inhibited galactose uptake in a non-competitive way, and decreased Vmax (302.1 nmol/100 mg w.w.) without modifying apparent Km (10.9 mM).

Na^+-dependent phenylalanine transport showed a higher affinity by its subs-

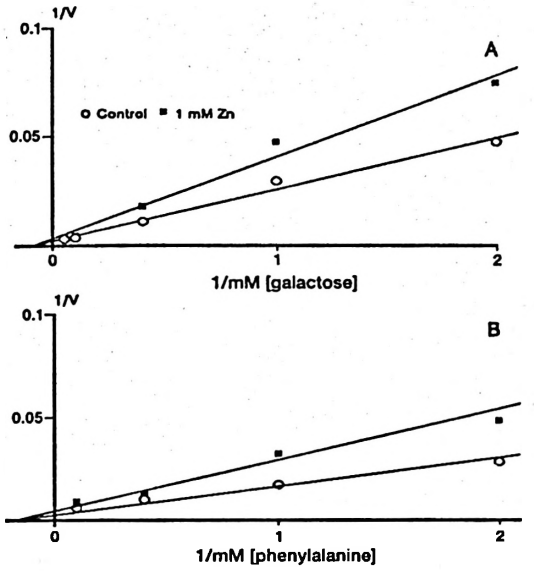

Fig. 4. *Lineaweaver-Burk plot of the inhibition of Na^+-dependent galactose and phenylalanine transport by 1 mM zinc.*
Galactose transport (A) was estimated from the difference between total uptake and that measured in the presence of 0.5 mM phlorizin. Na^+- dependent phenylalanine transport (B) was calculated as the difference between the respective uptake in the presence or absence of Na^+ in the incubation medium. Each value represents the difference between the means obtained from nine intestinal rings used per each sustrate concentration. V, nmoles galactose or phenylalanine/100 mg wet weigh. The linear regression analyses were used for the estimation of kinetic parameters.

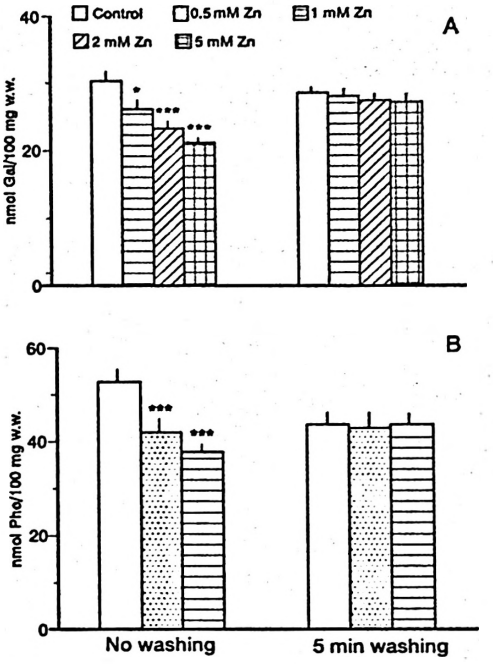

Fig. 5. *Reversibility of galactose and phenylalanine uptake inhibition by zinc.*
The intestinal rings were preincubated for 2 min without (Control) or with several Zn^{2+} concentrations in the incubation medium. Then, the rings were incubated with 0.5 mM galactose (A) or 0.5 mM phenylalanine (B) without intermediate washing or with 5 min washing in physiological solution KRT. Each bar represents the mean ± SEM of nine experimental values. * $p < 0.05$; *** $p < 0.001$ vs control.

trate than galactose one, with an apparent Km of 2.1 mM. However, the value of apparent Vmax obtained for phenylalanine transport was lower (181.8 nmol/100 mg w.w.). One mM Zn^{2+} also inhibited phenylalanine transport in a non-competitive manner, with modification of Vmax (136.6 nmol/100 mg w.w.) and hardly any change in Km (2.9 mM).

Reversibility of the zinc inhibitory effect on galactose and phenylalanine uptake.– After preincubation of the intestinal rings for 2 min in the presence of 1, 2 or 5 mM Zn^{2+} and following incubation in the absence of the metal, an inhibition of 0.5 mM galactose uptake by Zn^{2+} was observed (fig. 5 A). In these experimental conditions 1 mM Zn^{2+} inhibited galactose uptake in a lower degree (14 %) than the obtained when the metal was also present during the incubation time (25 %). Nevertheless, the washing of the intestinal rings during 5 min with KRT solution completely reversed zinc inhibitory effect.

Similarly to what happened with galactose, the inhihition of phenylalanine

uptake by preincubation with 0.5 or 1 mM Zn^{2+} was reversed by 5 min washing with KRT (fig. 5 B).

Discussion

Heavy metals are potent inhibitors of transport processes in many species and tissues. The results of the present study indicate that $ZnCl_2$ reduces galactose and phenylalanine uptake in everted rings of rat jejunum incubated for 2 min. Zinc affected galactose uptake in many ways like the phenylalanine uptake, although the latter was more sensitive to the metal than the former. Zn^{2+} inhibited the sodium-dependent transport of both substrates in a dose-dependent way and the inhibition seems to be due to the interaction between the metal and the Na^+-substrate cotransport mechanisms. Similar results were obtained for cadmium (13), chemically related to zinc, mercury (14, 19) and copper (20) on galactose and phenylalanine uptake in rat intestine *in vivo* and *in vitro*.

Uptake inhibition of both substrates increased with the Zn^{2+} concentration but not with the time of metal exposure to the tissue. Moreover, the inhibition was completely reversed by washing the intestinal rings with the physiological solution KRT. It is important to remark that after preincubation of the intestinal rings for 2 min in the presence of Zn^{2+} and subsequent incubation in its absence, a significant inhibition of galactose and phenylalanine uptake was observed. These results suggest that the metal interacts with chemical groups located at the luminal side of the mucosal membrane, probably with a low affinity for the metal. RODRÍGUEZ-YOLDI *et al.* also described a dose-dependent inhibition for Zn^{2+} on galactose (21) and threonine (22) accumulation in the rabbit jejunum *in vitro*, the inhibition not being increased by preincubation with zinc. However, WATKINS *et al.* (30) reported that zinc inhibition of glucose uptake by brush border membrane vesicles from pig intestine was a function of the time of incubation with the metal. In this case, the effect of zinc could result from interaction with ligands inside the vesicles. Thus, TACNET *et al.* (26) described several zinc binding sites in brush border membrane vesicles isolated from pig intestine: a first class of high-affinity extravesicular zinc binding sites, a second class of extravesicular sites with a very low affinity and intravesicular sites with a high affinity for zinc. Zinc as well as cadmium could probably be associated in the intestinal tract to intra or extracellular sites related with metal absorption or with sugar or amino acid transport systems (6). The accessibility of Zn^{2+} to these sites might differ with the experimental conditions.

Inhibition was exclusively exerted on mediated transport processes as no evidence was found for any change in the passive permeability of the tissue. Similar results have been previously reported for other heavy metals and for Zn^{2+} (15, 21, 22, 30). The inhibition produced by Zn^{2+} was evident only in the presence of sodium in the incubation medium, suggesting that the metal affected exclusively the sodium-dependent substrate transport systems.

These findings are consistent with two possible mechanisms of action for Zn^{2+}: a direct interaction with chemical groups related to the substrate-Na contransporters or an alteration of the sodium gradient across the luminal membrane through the ATPase inhibition. In published papers it has been described that zinc or other heavy metals (4, 9, 30) do not affect Na influx and an inhibition of Na/K

ATPase by Zn has been reported in renal epithelium (18). In our results the inhibition of galactose and phenylalanine transport cannot be attributed to a possible ATPase inhibition because of the short incubation period and the low absorption rate of the metal that hinders its accessibility to the enterocyte basolateral membrane. The fast reversion obtained by washing the intestinal rings with KRT also supports that suggestion. Hence, zinc appears to interfere with chemical groups in the luminal membrane directly or indirectly related with the substrate-Na^+ cotransporters.

On the other hand, zinc acts like a non-competitive inhibitor of the Na^+-galactose and Na^+-phenylalanine cotransporters. These results indicate that zinc modifies the functionality of the transporter without changing the affinity to its substrate. We obtained a similar non-competitive inhibition for cadmium (13), mercury (14) and copper (20) on galactose uptake *in vitro*. Nevertheless, the effect of heavy metals on kinetic parameters reported by different authors differs depending on the studied species or the experimental conditions (16, 21, 22, 24, 28, 30).

In summary, our results show that zinc inhibits the intestinal uptake of galactose and phenylalanine by impairment of the Na^+-substrate cotransport systems. Recent progress has been made for the characterization of the intestinal brush border Na^+/glucose contransporter (7, 27, 31). Tyrosyl residues, lysine residues, carboxyl groups and -SH groups of proteins and phosphate groups of phospholipids seem to play an important role (17, 18, 31). It is well known that zinc has a high affinity to thiol groups at physiologic conditions and can associate to metallothionein and other proteins through thiolate bound to cysteine residues (8, 13). Intestinal cadmium toxicity is thought to block physiologically essential sulfhydryl groups (2). Similarly, zinc might interact with sugar or amino acid cotransporters through binding to -SH groups, although a bind to other chemical groups cannot be discarded.

Acknowledgements

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Resumen

A. LUGEA, A. BARBER y F. PONZ. *Efecto del zinc sobre la absorción de galactosa y fenilalanina en intestino de rata* in vitro. Rev. esp. Fisiol. (J. Physiol. Biochem.), 51 (3), 139-146, 1995.

Se estudia el efecto del Zn^{2+} sobre la acumulación de galactosa y fenilalanina en anillos de yeyuno evertido de rata tras 2 min de incubación en solución fisiológica (KRT) continuamente oxigenada. La presencia de Zn inhibe la entrada de los sustratos en función de la concentración del catión, pero no del tiempo de exposición. A concentración 0,5 mM, el Zn^{2+} inhibe la captación de fenilalanina sin afectar la de galactosa, que se hace patente a 1 mM. La inhibición por Zn se ejerce sobre el transporte de galactosa y de fenilalanina dependiente de Na^+, sin modificar la entrada difusional de ambos sustratos o la del aminoácido independiente de Na^+. El transporte de galactosa y el de fenilalanina se inhibe no competitivamente por Zn, disminuyendo la Vmax y sin modificación de la Km. El lavado con solución KRT durante 5 min revierte totalmente el efecto inhibitorio del Zn sobre la absorción de los sustratos. Los resultados sugieren que el Zn ejerce su acción inhibitoria por unión reversible a grupos químicos, probablemente -SH, relacionados con los cotransportadores Na^+-sustrato de la membrana luminal de los enterocitos.

Palabras clave: Zn, Inhibición, Transporte intestinal, Azúcares, Fenilalanina.

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