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Zinc Transport in Human Fibroblasts: Kinetics and Effects of Ligands

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Introduction

Picolinic acid (PA), a tryptophan metabolite, is a bidentate chelating agent which appears to be active in zinc metabolism. A number of recent studies have shown that orally administered PA improves intestinal absorption of zinc in experimental animals (4, 9, 14, 17). Complexed with zinc, it decreased the zinc requirements of three patients with acrodermatitis enteropathica (AE) to one-third (2) and improved zinc absorption in men with pancreatic insufficiency as opposed to men with cirrhosis of the liver (1); moreover, the PA content of pancreatic extract appeared to be one explanation of the effectiveness of this medication in three patients with variant AE (13, 15). Several other studies have not shown an effect of PA at the intestinal site. The reason for these inconsistencies appears to be related to the zinc concentration of the medium. This was suggested by two recent studies, which demonstrate that PA and other ligands are effective in raising tissue or plasma zinc if dietary zinc intake is low (9, 14).

According to Rebello et al. (19), PA concentration in milk is similar to nicotinic acid, about 5 mcM. Using a different method, Evans et al. (4) obtained substantially higher values, especially in human milk. Concentration in plasma is 12 mcM (2, 15), sufficient to bind three-quarters of circulating zinc. This presence of PA in plasma suggests that it serves a function at sites other than the intestinal mucosa; moreover, certain observations in variant AE originally led us to speculate that PA facilitates zinc transport at peripheral tissue sites or enhances zinc binding to metalloenzymes (12). We studied therefore the effect of PA when added to tissue culture of diploid fibroblasts. This required characterization of the zinc transport mechanism in human fibroblasts, which had not been done. Our methodology of using fibroblasts grown on coverslips for transport studies has been used before only in the study of amino acid transport (8).

The results of our zinc transport studies and of the effect of PA and other ligands form the basis of this report. Whereas PA seems to enhance the uptake of zinc by the intestinal mucosa under certain conditions (4, 14, 17), we found that it inhibits transport of zinc into fibroblasts.

Materials and Methods

Human diploid fibroblasts from fetal tissue (subcultures limited to less than 12) were grown in Eagles essential medium (25), containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mcg/ml). Transport studies were conducted on cells grown on washed sterile coverslips (11 x 22 mm) (26). The seeding and incubation procedure was essentially that of Foster and Pardee (8). With fibroblasts near confluency, coverslips were rinsed twice with phosphate-buffered saline-glucose (PBSG) (27), containing 130 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, one mM $CaCl_2$, 5 mM glucose and 10 mM Na_2HPO_4 (pH 7.4). The coverslips were left in the final PBSG wash for one h at 37°C (8). It was assumed that this step minimizes endogenous zinc concentration. Cell viability appears unaffected (8). Because maximum density of monolayered cells may decrease the accumulation rate of essential nutrients we utilized coverslips with evenly distributed cells that had not quite reached confluency. Some interassay variability of zinc uptake was probably due to inability to duplicate the state of confluency. The incubation medium for the transport studies contained PBSG, $ZnCl_2$ (5 to 40 mcM) and 0.05 uCi/ml of [^{65}Zn] (30), in a total volume of 25 ml. Incubations were carried out at 37°C for time periods ranging from 30 sec to 20 min, using 50 ml disposable polypropylene beakers (28) and a holding rack (29). After incubation, coverslips were dipped six times into separate solutions of cold saline, using a holding rack. Drained coverslips were placed separately into glass scintillation vials. After mixture with 75 mcl distilled water and 400 mcl soluene-350 (31), fibroblasts were solubilized by heating for 30 - 45 min at 45°C. Scintillation fluid (32) (10 ml)was added and radioactivity counted in a Packard Tricarb Liquid Scintillation Spectrometer, Model 3385 (31), efficiency 36%. Zinc uptake by fibroblasts was calculated from the specific radioactivity of the zinc in the medium. Zinc uptake was related to the average protein content per coverslip, which was determined for each assay from several coverslips of each culture disk. The protein content, which was shown to reflect cell number (8), ranged between 50 - 80 mcg per coverslip. The effect of PA on zinc uptake by fibroblasts was studied by adding PA to the incubation mixtures in concentrations ranging from 20 - 100 mcM. The effect of other zinc binding ligands, citric acid, and histidine (100 mcM), and of metabolic inhibitors, KCN (one mM) and ouabain (one mM), was also studied. Protein was determined by the Lowry modification of the Folin-Ciocalteu method (16).

Results

In studies relating zinc uptake to the time of incubation, rapid uptake occurred during the first 2 min (phase I) and was followed by slower uptake (phase II). The rate of zinc uptake increased with increasing zinc concentrations in the medium (Fig. 1). At concentrations of 20 - 40 mcM, zinc accumulation continued to increase throughout the entire incubation period up to a maximum to 5 nmoles per mg protein. After 20 min incubation, saturation was not achieved at the highest zinc concentration tested (40 mcM). By contrast, at a concentration of 5 mcM, zinc accumulation leveled off at one nmoles per mg protein after only 1 to 2 min of incubation.

The relation between substrate concentrations and the velocity of uptake was curvilinear during phase I of incubation (0 - 2 min). A drastic change in the early part of phase II resulted in a near linear correlation for the period between 2 - 10 min of incubation. These findings are indicative of facilitated uptake during phase I.

The possible involvement of an active, energy requiring transport mechanism during any stage of zinc uptake was tested by incubating the cells in the presence of KCN (one mM). In two separate experiments we observed during the first 2 min of incubation 60 and 62% inhibition of zinc uptake, respectively. During two longer incubation periods, starting at 2 min and up to 10 and 20 min, respectively, KCN inhibition was 26%, each (Table 1). Zinc uptake was not affected by the addition of ouabain (one mM) to the incubation medium.

Apparent Km and Vmax values for zinc transport were determined from double reciprocal plots, using short time incubations of 1 and 2.5 min. For 1.0-min incubation, Km was 8.3 mcM and Vmax 7.7 nmoles/mg protein/min. For 2.5 min incubation Km was 9.3 mcM; Vmax was 2.3 nmoles/mg protein/min and decreased further with prolongation of the incubation period.

The inhibitory effect of ligands on zinc uptake was compared at a zinc:ligand ratio of 1:10 (Fig. 3). Inhibition by PA was greater on an equimolar basis than that of citric acid or histidine. The inhibitory effect of PA at the 1:10 ratio was the same during phase I and II of zinc uptake (60%). At low PA concentrations, inhibition of phase II uptake was in a similar high range whereas phase I uptake was only minimally affected, 7% inhibition at a zinc: ligand ratio of 1:2 (Table 2). A Dixon plot was constructed, using 5-min incubations and PA as the inhibitor in concentrations of 20 - 80 mcM. Inhibition was linear over the indicated PA concentrations range (Ki 12.5 mcM).

Discussion

Detailed studies of the kinetics of zinc transport have been performed in the past in hepatocytes (7, 22), HeLa cells (3), leucocytes (11), lymphocytes (18), and microorganisms (6). Mouse fibroblasts were used by Schwarz and Matrone (20), who studied the effect of amino acids, albumin, and citrate on zinc transport. Sugarman and Munro (24) used human fibroblasts when they tested the effect of age on zinc accumulation. The kinetics of the uptake mechanism were not investigated in either of these fibroblast studies. It appears, therefore, that our report is the first detailed investigation using human fibroblasts. Because this tissue is readily accessible to humans, it may become useful in investigations of genetic disorders of trace metal metabolism.

Our findings show that the mechanism of uptake is similar to that in hepatocytes (7, 22) and leukocytes (11), in that it occurred in two phases, one early phase of rapid uptake, followed by a phase of slower uptake. The relation between the velocity of uptake and substrate concentration indicates that the early phase is saturable and, therefore, apparently carrier-mediated. Zinc uptake during the second phase appears to be passive.

Data on the effect of metabolic inhibitors on zinc transport in hepatocytes is conflicting. Failla and Cousins (7) showed that inhibitors of oxidative metabolism decreased the amount of accumulated zinc, whereas Stacey and Klaassen found no effect by KCN (22). In our studies, KCN inhibited zinc uptake by 62% during the first 2 min. The effect tapered off after the early phase to 26% between 2 - 10 min of incubation, with no further increment in inhibition after 10 min. These reported inconsistencies have been attributed to the ability of the CN group to bind zinc, however, analogous to the PA effect, KCN inhibition should have affected phase II more than phase I if this were the case. Ouabain had no inhibitory effect, indicating that zinc transport does not require ATP, a fact which is compatible with operation of a carrier-mediated transport mechanism during phase I.

PA acted (in vitro) as a powerful inhibitor of zinc uptake, especially during phase II, when a Zn:PA ratio of one:two caused 75% inhibition. In general, inhibition of passive uptake suggests that the inhibitor does not compete with a carrier. This appears to be the case with PA, which is a known chelating agent. The inhibitory effect of PA during phase I thus may be expressed as "competition" between PA and the carrier for free zinc in the medium, with which PA forms a stable complex. Such "quasi" inhibition may explain why we were able to obtain on the Dixon plot an inhibitory constant, indicative of a competitive mode of inhibition. An alternate, though less likely explanation, is the existance of "true" competition between zinc and the Zn:PA complex for the carrier.

Assuming a physiologic role for PA, these data show that it's effect would be entirely different at fibroblast membranes of peripheral tissues than the effect observed by some investigators at intestinal transport sites. Zn:PA complex given orally to a patient with AE (2), variant AE (12, 13) and pancreative insufficiency (1) improved zinc absorption. Uncomplexed PA given orally to rats also improved intestinal absorption of zinc (4, 9, 14, 17) although the effect may be limited to low zinc concentrations (9, 17). Our studies now show that the characteristics of the Zn:PA complex make PA suitable to function both as a compound that promotes zinc absorption and prevents entry into intestinal tissues after it is absorbed. As such, PA could facilitate distribution of zinc and promote its conservation (5).

Whether PA functions indeed as a trace metal carrier in body fluids is not known, because current methods for measuring PA do not distinguish between free and complexed PA and different investigators disagree over the actual amount of PA that they found in physiologic fluids, mainly human milk (4, 19) and pancreatic excretions (10, 13). Hurley and Lönnerdal (10) therefore believe that the effect of PA is nonspecific and metabolically unimportant. This study of the in vitro effect of PA on fibroblast uptake of zinc shows that PA may be important as a ligand, along with others, if it is present in plasma in the amounts indicated. A more important role may be anticipated in the kidney, providing the zinc-PA complex is filtered through the glomerulus, especially because albumin is not filtered and PA is a stronger ligand than histidine, generally considered to be the strongest among amino acids. Assuming similar characteristics of the Zn:PA complex at the renal tubular site as in intestine, PA could be responsible for the reabsorption of zinc. A role for PA in renal control of zinc metabolism is also suggested by the finding of disproportionately high activities of picolinic acid carboxylase, the enzyme responsible for PA formation, in renal tissue (23).

Addition of uncomplexed PA to food did not affect zinc absorption in our patients with AE although the feeding of Zn:PA complex was effective (2). Similar observations were made in adult volunteers who were given either PA in aqueous solutions or with meals along with pharmacological doses of zinc (21). But when the same amount of zinc was given in aqueous solutions along with ferrous ion, addition of PA improved absorption of pharmacological doses of zinc (Zn:PA ratio of 1:6). It therefore may be anticipated that the relation between PA,competetive trace metals and other ligands, like albumin and amino acids, is as complex in plasma and interstitial fluids as at the intestinal transfer site

Because our system did not contain other ligands, known to be present in plasma and interstitial fluids, only limited conclusions can be drawn from this study. In the absence of these ligands, phase I is rapidly saturated at physiologic zinc concentrations of 10 - 20 mcM, whereas phase II uptake continues for considerable time longer than tested in our incubation experiments. Assuming that the true plasma Zn:PA ratio is somewhat higher than the 1:2 ratio tested in our system, we can expect negligible inhibition during phase I, but a considerable effect during phase II, which may be important for zinc distribution following intestinal absorption and uptake into the portal system. Further studies are necessary to explore these complex interactions.

Summary

The kinetics of zinc transport were studied in human diploid fibroblasts grown on coverslips. Zinc uptake was rapid during the first 2 min of incubation (phase I) and was followed by slower uptake (phase II). Zinc uptake was positively correlated with zinc concentrations in the medium. The relation between zinc concentration and velocity of uptake was curvilinear during phase I. A

drastic change during the early part of phase II resulted in a near linear correlation for the period between 2 - 10 min of incubation. The metabolic inhibitor KCN (one mM) affected mainly phase I and resulted in 62% inhibition. Ouabain had no inhibitory effect. These findings are indicative of facilitated uptake during phase I, when a carrier-mediated, energy-requiring transport mechanism seems to be operative. Km values were 8.3 and 9.3 mcM for the first 1.0 and 2.5 min incubation, respectively. Corresponding values for Vmax were 7.7 and 2.3 nmoles/mg protein/min. Addition of PA to the incubation medium at a Zn:PA ratio of 1:10 resulted in about 60% inhibition during both phases of zinc uptake. At a Zn:PA ratio of 1:2, inhibition was considerably less during phase I than II (7% and 75%, respectively). The inhibition was positively related to PA concentrations Of 20 - 80 mcM; Ki 12.5 mcM. PA was a more potent inhibitor on an equimolar basis than either citrate or histidine.

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26. Arthur H. Thomas Co., Philadelphia, PA.
27. Unless otherwise stated all chemicals were obtained from Sigma Chemical Co., St. Louis, MO.
28. Fisher Scientific Co., Pittsburgh, PA.
29. Lipshaw Co., Detroit, MI.
30. [^{65}Zn], carrier free, accelerator produced: New England Nuclear, Boston, MA.
31. Packard Instruments, Co., Downers Grove, IL.
32. Liquifluor, New England Nuclear, Boston, MA.: (42 ml of liquifluor diluted to one liter with scintillation toluene).

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TABLE I

Effect of KCN on the velocity of zinc uptake[1]

Experiment	Incubation time (min)	Zinc uptake velocity (nmoles/mg protein/min) Control	plus KCN	Inhibition (%)
I.	0 to 2	0.420	0.160	62
	2 to 10	0.074	0.055	26
II.	0 to 2	0.700	0.280	60
	2 to 20	0.190	0.140	26

1. The coverslips with fibroblasts were incubated in duplicates for 2, 10, and 20 min. The medium contained 10 mcM zinc and [^{65}Zn] tracer (controls). KCN concentration was one mM. The velocity of uptake was calculated from the specific radioactivity of [^{65}Zn] in the medium.

TABLE II

Inhibition of zinc uptake by picolinic acid during the active and passive phase of zinc transport

Incubation time (min)	Percent inhibition at 1:1 zinc:picolinic acid ratio	1:6	1:10 of the medium
0 to 2.5 (phase I)	7	38	59
2.5 to 10 (phase II)	75	85	60

1. Coverslips with fibroblasts were incubated for the time periods specified. Each point is the mean of four coverslips. The medium contained 10 mcM zinc and [^{65}Zn] tracer; picolinic acid was added as specified by the ratios.

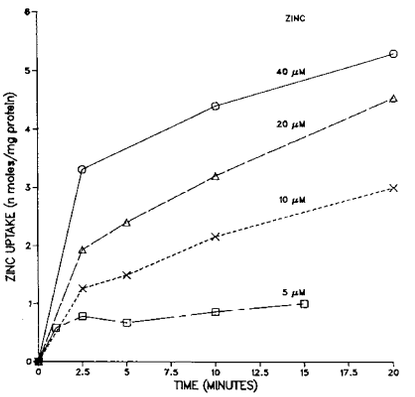

Fig. 1 Zinc uptake by skin fibroblasts, grown on coverslips, in relation to incubation time. The zinc concentration in the medium ranged from 5 to 40 mcM. Each point represents a mean of four coverslips.

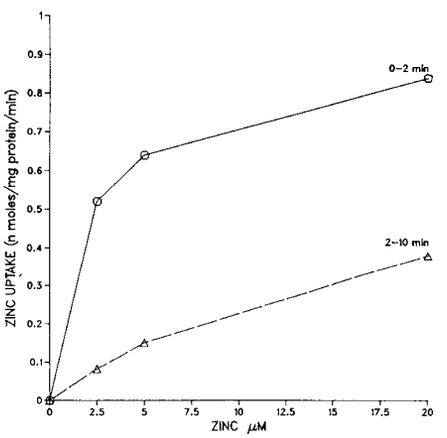

Fig. 2 Velocity of zinc uptake by skin fibroblasts in relation to zinc concentration in the medium. Incubation periods were either 0 to 2 or 2 to 10 min. Each point represents an average of two coverslips.

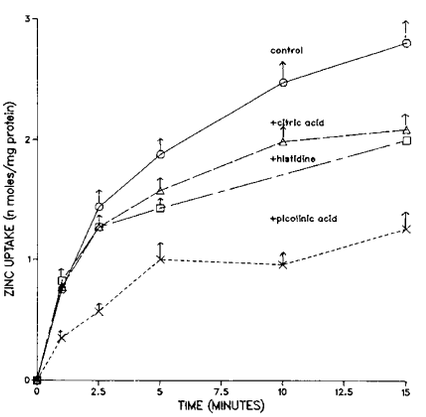

Fig. 3 Effects of ligands on zinc uptake by skin fibroblasts. Zinc concentration in the medium, was 10 mcM and the concentration of the ligands was 100 mcM. Each point represents the mean of four coverslips and +1 S.D. as indicated by arrow.

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