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Zinc uptake into fibroblasts is inhibited by probenecid

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Abstract

Cellular zinc transport has not been fully characterized. The role of an anion carrier was investigated by treating normal human fibroblasts, and those carrying a mutation which affects zinc transport, *acrodermatitis enteropathica* (AE), with the anion carrier inhibitor, probenecid. Zinc uptake (2, 10, or 20 μmol l^{-1} ^{65}zinc) was determined during initial rates of uptake (15 min) following treatment with 0, 10 or 20 mmol l^{-1} probenecid. Probenecid stimulated extracellular zinc binding in normal and AE fibroblasts. Probenecid inhibited the internalization of zinc in normal, but not AE, fibroblasts. Normal fibroblasts exhibited an apparent K_m which was reduced by 53% and 44% in the 10 and 20 mmol l^{-1} probenecid treated cells. The V_{max} was also reduced in the normal fibroblasts by 51% and 50% in the 10 and 20 mmol l^{-1} probenecid treated cells. The results suggest that a probenecid-sensitive anion carrier is involved in the internalization of zinc in human fibroblasts. The lack of an effect of probenecid on the internalization of zinc in the AE fibroblasts suggests that the mutation involves a probenecid-sensitive anion transport system, and that there may be a secondary mechanism for zinc transport in these cells. © 1998 Elsevier Science B.V.

Keywords: Probenecid; Zinc; Transport; *Acrodermatitis enteropathica*

1. Introduction

The cellular mechanism for zinc uptake has not been fully characterized. One aspect of cellular zinc uptake appears to involve an anion carrier system. Zinc uptake into human erythrocytes is stimulated by bicarbonate and chloride, and inhibited by the band 3 anion exchange inhibitor 4,4′-diiosthiocyanatostilbene-2,2′-disulphonic acid (DIDS) [1,2]. The transported complex has been proposed to be $[Zn(HCO_3)_2Cl]^-$ [1], $ZnCO_3Cl^-$ or $[Zn(HCO_3)Cl \cdot OH]^-$ [2]. Bicarbonate also stimulates zinc uptake into human fibroblasts [3]. Brush border membrane vesicles from pig small intestine also appear to transport zinc via an anion carrier system, although a thiocyanate complex was the anionic species [4–6]. However, this mechanism was not DIDS sensitive [6].

Anion transport mechanisms other than those sensitive to DIDS have been identified. These include α-cyano-4-hydroxycinnamate [7] and ρ-[dipropylsulfamoyl]benzoic acid (probenecid) [8–10]. The mechanism of inhibition of these inhibitors on cellular zinc transport has not been investigated. Probenecid inhibits cellular folate and methotrexate transport by L1210 and MA104 cells [9,10]. Furthermore, a

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probenecid-sensitive anion transport mechanism has been proposed in the model for non-clathrin coated pit vesicular transport, termed potocytosis [11,12]. We have previously reported that nystatin, a sterol-binding antibiotic that inhibits the formation of caveolae (the vesicles of potocytosis), also inhibits zinc transport [13]. If zinc transport occurs via potocytosis, it may also be inhibited by probenecid. Thus, we hypothesized that zinc transport would be inhibited by probenecid. Further, we also hypothesized that probenecid would inhibit further the poor zinc uptake by fibroblasts containing the *acrodermatitis enteropathica* mutation. We used these cells because we hypothesized that more than one mechanism might exist for zinc transport. Our results support these hypotheses. We found that cellular zinc transport occurs through a probenecid-sensitive anion carrier, suggesting that potocytosis is involved. However, the results also suggest the existence of a secondary zinc uptake system. The AE mutation significantly inhibited zinc uptake but probenecid treatment did not further reduce zinc transport in these cells.

2. Materials and methods

2.1. Cell culture

Fibroblasts were purchased from the Coriell Institute for Medical Research Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were from Caucasian males. The normal fibroblasts (GM5659B) were obtained from a 14 month old infant. *Acrodermatitis enteropathica* (GM2814) fibroblasts were from a 6 month old infant. The cells were grown in minimal essential medium (Eagle's) (GIBCO–BRL, Grand Island, NY) containing 0.01 mg l^{-1} gentamicin, 0.06 g l^{-1} penicillin, 0.1 g l^{-1} streptomycin, 20% uninactivated fetal calf serum and double the normal concentrations of essential and non-essential amino acids and vitamins. The growth medium contained approximately 16 μmol l^{-1} zinc as measured by atomic absorption spectroscopy.

2.2. Zinc transport

The fibroblasts were subcultured in 3.83 cm^2 dishes (22 mm diameter) at 1.3×10^4 cells cm^{-2} and grown for 4 days. On the fourth day, the growth medium was removed and the cells rinsed twice with HGS buffer (10 mmol l^{-1} HEPES, 138.3 mmol l^{-1} NaCl, 7 mmol l^{-1} KCl, 5.6 mmol l^{-1} dextrose, 1.3 mmol l^{-1} $CaCl_2$, pH 7.4) at 37°C. The cells were then incubated at 37°C for 20 min with HGS buffer or buffer containing 10 or 20 mmol l^{-1} probenecid, pH 7.5. These concentrations of probenecid were chosen because 10 mmol l^{-1} has been shown to inhibit folate transport in MA104 cells [10]. The effect on zinc transport of 20 mmol l^{-1} probenecid was investigated to determine whether maximal inhibition of zinc transport was obtained at 10 mmol l^{-1} probenecid.

The incubation buffer was removed and the cells were incubated for 15 min with fresh buffers at 37°C containing 2 to 20 μmol l^{-1} ^{65}zinc (1 kBq). Previous results indicated that zinc uptake in these fibroblasts was linear between 10 and 30 min [22], indicating that the 15 min time point is within the initial rate for zinc uptake. Carrier-free ^{65}zinc was used as a tracer (Amersham, Arlington Heights, IL). Transport was stopped by placing the culture dishes on ice. The labeled buffer was removed and the fibroblasts were rinsed with 1 ml ice-cold EDTA buffer (10 mmol l^{-1} HEPES, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} EDTA, pH 7.4) for 30 s. The reaction was stopped with the addition of 1 ml ice-cold HGS buffer. The buffers were removed and the cells rinsed with 1 ml ice-cold HGS. This buffer was removed and the cells were rinsed for 30 s with ice-cold acidic saline (150 mmol l^{-1} NaCl, 40 mmol l^{-1} acetic acid, pH 3.0). The reaction was stopped by the addition of 1 ml ice-cold HGS. This rinse was removed and placed in scintillation vials. The HGS rinse was repeated and also placed with the acidic saline rinse, and the radioactivity of the solution determined by gamma counting. Preliminary experiments indicated that a single EDTA rinse removed 15.2% of the cell-associated label, compared to 17.6% of the cell-associated label after 4 EDTA rinses. A single acidic saline rinse removed 17.2% of the label compared to 19.29% of the label removed after four acidic saline rinses. However, a single rinse with EDTA followed by a single rinse with acidic saline removed 19.8% of the cell-associated zinc. Therefore, this method was chosen for determining externally bound and internalized zinc.

The cells were lysed by adding 0.2 M NaOH–0.2%

sodium dodecyl sulfate for 15 min at room temperature and freezing overnight at −20°C. The lysate was thawed and the radioactivity determined by gamma counting. An aliquot was used for protein determination with the bicinchoninic acid method [21]. The amount of zinc present in the acidic saline rinse represents externally-bound, acid-sensitive cellular zinc. The amount of zinc present in the NaOH–SDS solution represents internalized, acid-resistant zinc.

2.3. Data analysis and statistics

The data were analyzed by the Lineweaver–Burke double reciprocal plot. The intercepts of the *x*-axis or *y*-axis were used to determine the apparent K_m and V_{max}, respectively. Significance was determined by ANOVA and Student–Neuman–Keuls unpaired *t*-test (Statmost™ 1.01, DataMost, Salt Lake City, UT). Differences were considered significant when $p < 0.05$.

3. Results

The accumulation of zinc by human fibroblasts, its binding to external binding sites, and its transport into human fibroblasts appeared to begin to saturate at 20 μmol l^{-1} zinc (Figs. 1 and 2). The exception was Fig. 2C, in which zinc transport in the AE fibroblasts appeared linear at the zinc concentrations used. The total cell-associated zinc (Fig. 1A and Fig. 2A) was divided into an acid-sensitive, externally bound zinc compartment (Fig. 1B and Fig. 2B) and an acid-resistant, internalized zinc compartment (Fig. 1C and Fig. 2C).

Treatment of normal fibroblasts with 10 or 20 mmol l^{-1} probenecid did not affect the total cell-associated zinc (Fig. 1A). Probenecid at a concentration of 10 mmol l^{-1} significantly increased the accumulation of zinc in the external compartment at 2 μmol l^{-1} zinc (Fig. 1B) by 25% over normal levels, but not at any other zinc concentration. Probenecid at a concentration of 20 mmol l^{-1} significantly increased the accumulation of zinc in the external compartment at 2 μmol l^{-1} zinc by 44%, and at 5 to 20 μmol l^{-1} zinc by 29% to 33%, over normal levels (Fig. 1B). Increasing the probenecid concentration from 10 to 20 mmol l^{-1} significantly increased the accumulation of zinc in the external compartment of these cells at 5 and 10 μmol l^{-1} zinc by 33% and 20%, respectively.

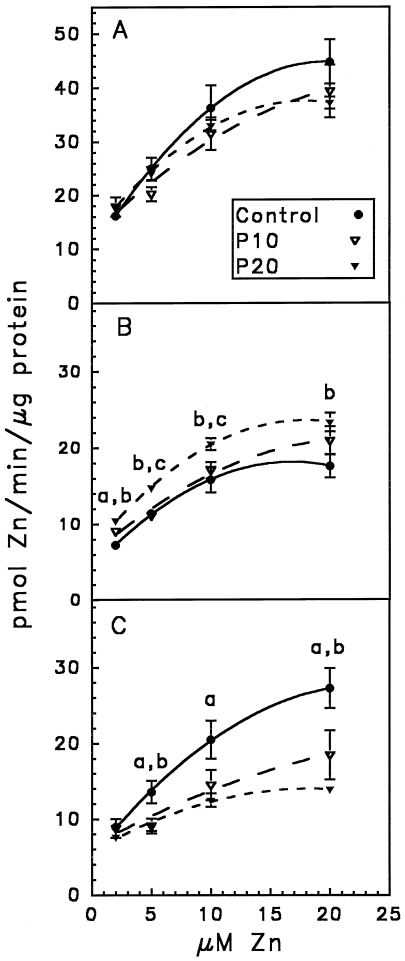

Fig. 1. The effect of probenecid on zinc transport in normal human fibroblasts. Zinc transport was measured in normal fibroblasts treated with 10 or 20 mmol l^{-1} probenecid. Each data point represents the mean ± SE ($n = 9$). Statistical analysis was performed by the Student's *t*-test. The data points which are significantly different from each other ($P < 0.05$) are indicated by *a*, *b*, or *c* (*a*: control vs. 10 mmol l^{-1} probenecid; *b*: control vs. 20 mmol l^{-1} probenecid; *c*: 10 mmol l^{-1} probenecid vs. 20 mmol l^{-1} probenecid). The concentrations of probenecid are designated *P10* (10 mmol l^{-1} probenecid) and *P20* (20 mmol l^{-1} probenecid). (A) Total cell-associated ^{65}zinc. These data include zinc in the acid-sensitive and the acid-resistant compartments. (B) Externally bound, acid-sensitive ^{65}zinc. (C) Internalized, acid-resistant ^{65}zinc.

AE fibroblasts treated with probenecid exhibited significant increases in total cell-associated zinc at all zinc concentrations (Fig. 2A). Zinc accumulation in the external compartment was significant from 2 to

20 μmol l^{-1} zinc regardless of the probenecid concentration, and exhibited an increase of 58% to 88% above control levels. The accumulation of zinc in the external compartment was not significantly increased further by increasing the probenecid concentration from 10 to 20 mmol l^{-1}.

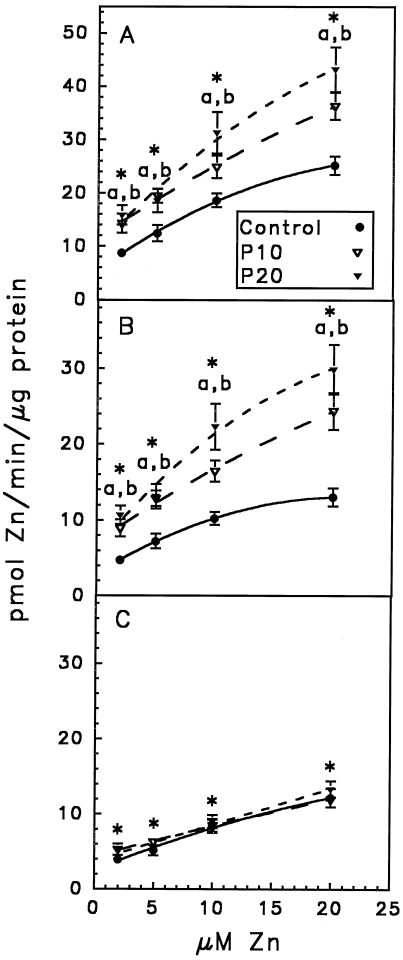

Fig. 2. The effect of probenecid on zinc transport in *acrodermatitis enteropathica* (AE) fibroblasts. Zinc transport was measured in AE fibroblasts treated with 10 or 20 mmol l^{-1} probenecid. Each data point represents the mean ± SE ($n = 9$). Statistical analysis was performed by the Student's *t*-test. The data points which are significantly different from each other ($P < 0.05$) are indicated by *a* and *b* (*a*: control vs. 10 mmol l^{-1} probenecid; *b*: control vs. 20 mmol l^{-1} probenecid). The concentrations of probenecid are designated *P10* (10 mmol l^{-1} probenecid) and *P20* (20 mmol l^{-1} probenecid). The control AE data points which are significantly different from the control normal data points are indicated by an asterisk (*). (A) Total cell-associated ^{65}zinc. These data include zinc in the acid-sensitive and the acid-resistant compartments. (B) Externally bound, acid-sensitive ^{65}zinc. (C) Internalized, acid-resistant ^{65}zinc.

Table 1
Apparent K_m and V_{max} values of normal fibroblasts treated with probenecid

	0 mM	10 mM	20 mM
K_m	5.19 ± 0.87^{a}	$2.44 \pm 0.46^{a,b}$	$2.93 \pm 0.91^{a,c}$
V_{max}	32.36 ± 5.23^{a}	$15.83 \pm 1.56^{a,b}$	$16.05 \pm 1.79^{a,c}$

The data are expressed as the mean ± SE. The K_m and V_{max} were determined by fitting the curves generated from the data using the Lineweaver–Burke double reciprocal plot. The mean values for the data points in Fig. 1C were also analyzed by a direct linear plot, which indicated decreases in K_m and V_{max} with probenecid treatment as well. Statistical analysis was performed on the K_m and V_{max} values by ANOVA using the Student–Neuman–Keuls method. The units for the apparent K_m are μM. The units for the V_{max} are pmol/min/μg protein. For each kinetic parameter, values with the same superscripts are significantly different from each other ($P < 0.05$).

The internalization of zinc in normal fibroblasts (Fig. 1C) was significantly reduced in the 10 mmol l^{-1} probenecid treated cells at zinc concentrations of 5 and 20 μmol l^{-1} by 34% and 32%, respectively. Normal cells treated with 20 mmol l^{-1} probenecid exhibited reduced zinc accumulation in their internal compartment at zinc concentrations from 5 to 20 μmol $^{-1}$ (by 32% to 49%, respectively) when compared to untreated control cells. Probenecid treatment of the AE fibroblasts did not affect the accumulation of zinc in their internal compartment (Fig. 2C). Zinc transport was affected by the AE mutation alone (cf. the results for the control data of Fig. 2 [AE cells] with Fig. 1 [normal cells]).

The apparent K_m and the V_{max} were determined from the data in Fig. 1C (Table 1). Normal fibroblasts exhibited a significant reduction in both kinetic parameters in the presence of probenecid. Probenecid treatment at 10 and 20 mmol l^{-1} reduced the apparent K_m by 53% and 44% compared to control, respectively. Probenecid treatment at 10 and 20 mmol l^{-1} also reduced the V_{max} by 51% and 50% compared to control, respectively.

4. Discussion

The hypothesis tested by the present work was that probenecid would inhibit zinc transport in normal and

AE fibroblasts. The results from normal fibroblasts support the hypothesis, and are consistent with cellular zinc transport involving an anion carrier system. Probenecid has been used to identify the role of anion transport in the cellular uptake of folate [10] and methotrexate [9,14–16], and may be associated with the vesicular transport mechanism called potocytosis [11].

However, probenecid treatment did not inhibit zinc transport in the AE fibroblasts any further than was accounted for by the mutation alone. The results from these cells suggest that the probenecid-sensitive anion transport system may be the defect caused by the AE mutation. These results suggest that a secondary zinc transport system is available in the AE cells. Though not compensatory, this system is apparently capable of transporting sufficient zinc for relatively normal cellular function. However, the AE fibroblasts do contain less intracellular zinc and exhibit a reduction in the activity of the zinc-dependent enzyme, 5′-nucleotidase, when compared with normal fibroblasts [22].

The V_{max} and the apparent K_m measured in the normal cells were affected by probenecid treatment to the same degree, indicating that uncompetitive inhibition was occurring [17]. Probenecid apparently blocks the anion carrier, thereby restricting the transport of zinc into the cell. The reduced transport appears to increase the availability of extracellular zinc-binding sites, as indicated by an increase in the presence of zinc in the acid-sensitive compartment in the probenecid-treated cells. It is not known whether probenecid forms a complex with zinc, thereby affecting its binding to the cell surface. However, if this were so one might expect similar reductions in the internalization of zinc with probenecid treatment regardless of the genotype of the cell. The results in Fig. 1C and Fig. 2C indicate that probenecid only inhibited zinc internalization in the normal fibroblasts, not in the AE fibroblasts.

The model of potocytosis for the transmembrane transport of folate, and for zinc, involves the formation of vesicles through a cholesterol-dependent process, the activation of a monensin-sensitive proton pump, and transport through a probenecid-sensitive anion carrier [11]. Previous results from this lab indicate that cellular zinc transport in fibroblasts is inhibited by nystatin [13], a membrane sterol-binding drug which also inhibits the formation of the potocytotic vesicles called caveolae [18]. Furthermore, the depletion of cellular cholesterol, i.e., the reduction in the number of functional caveolae, significantly reduces the cellular accumulation of folate and affects the internalization of the folate receptor [19,20]. The depletion of cellular cholesterol also reduces the cellular uptake of zinc (Grider, unpublished data). Therefore, the data support the hypothesis that potocytosis may be involved in the transmembrane transport of zinc.

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