Published in final edited form as:
Toxicol Appl Pharmacol. 2007 May 1; 220(3): 349–356. doi:10.1016/j.taap.2007.02.001.

ALUMINUM STIMULATES UPTAKE OF NON-TRANSFERRIN BOUND IRON AND TRANSFERRIN BOUND IRON IN HUMAN GLIAL CELLS

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Abstract

Aluminum and other trivalent metals were shown to stimulate uptake of transferrin bound iron and nontransferrin bound iron in erytholeukemia and hepatoma cells. Because of the association between aluminum and Alzheimer's Disease, and findings of higher levels of iron in Alzheimer's disease brains, the effects of aluminum on iron homeostasis were examined in a human glial cell line. Aluminum stimulated dose- and time-dependent uptake of nontransferrin bound iron and iron bound to transferrin. A transporter was likely involved in the uptake of nontransferrin iron because uptake reached saturation, was temperature-dependent, and attenuated by inhibitors of protein synthesis. Interestingly, the effects of aluminum were not blocked by inhibitors of RNA synthesis. Aluminum also decreased the amount of iron bound to ferritin though it did not affect levels of divalent metal transporter 1. These results suggest that aluminum disrupts iron homeostasis in the brain by several mechanisms including the transferrin receptor, a nontransferrin iron transporter, and ferritin.

Keywords

aluminum; iron; glial; transporters; transferrin

INTRODUCTION

The redox potential of iron is ideal for respiration but also potent for catalyzing the generation of reactive oxygen species. For these reasons, iron homeostasis is strictly regulated by several iron transport proteins and very little iron is observed not bound to macromolecules. When the cell needs more iron, the transferrin receptor (TfR) increases,

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allowing for more iron to be brought into the cell and the storage protein ferritin decreases enabling more iron to reach the respiratory chain and other iron requiring systems. Under iron replete conditions, TfR decrease and levels of ferritin increase allowing iron to be stored in a complex with ferritin, which prevents iron-mediated oxidative stress (Aisen *et al.*, 2001). In addition to nutritional needs, TfR and ferritin respond to other physiological stresses including hypoxia and infection (Vyoral and Petrak, 2005). Divalent metal transporter 1 (DMT1) is also involved in iron homeostasis. It is a proton-coupled metal transporter in the intestine that increases in response to low dietary iron (Gunshin *et al.*, 1997). Four DMT1 mRNA splice variants have been identified in various tissues and two display responsiveness to iron (Hubert and Hentze, 2002). DMT1 is also an intracellular iron transporter that is involved in vesicle trafficking when the transferrin iron complex binds to the TfR.

There is also evidence for uptake of iron not bound to transferrin (i.e. nontransferrin bound iron) outside of the intestine though its' significance is unclear. Evidence for uptake of nontransferrin bound iron includes the observation of iron accumulation in the liver of a genetic strain of mice deficient in transferrin and in genetic diseases when the level of iron in the serum is in excess of the transferrin binding capacity (Trenor *et al.*, 2000). Furthermore, some organs develop in TfR knockout mice during embryogenesis suggesting that iron transport in these organs did not require TfR (Levy *et al.*, 1999). DMT1 has been suggested to be the transporter for nontransferrin bound iron but the mechanisms is not understood. One concern is the source of protons because the extracellular fluid is usually at neutral pH.

The identity of the transporters for nontransferrin bound iron and other facets of iron homeostasis need more study because of the involvement of iron in disease. Recent studies have suggested that environmental toxicants impair health by disrupting iron homeostasis. For example, the deleterious effects of manganese on health might be due to oxidative stress and involve a disruption in iron homeostasis (Erikson *et al.*, 2006). Rats fed high levels of manganese display elevated levels of TfR mRNA and DMT1 in the brain (Garcia *et al.*, 2006; Li *et al.*, 2006). Manganese has been suggested to increase uptake of iron through the TfR (Zheng and Zhao, 2001) and affect iron homeostasis in PC12 cells.(Kwik-Uribe and Smith, 2006). Other metals have also been shown to disrupt iron homeostasis. Interestingly, aluminum and gallium have been shown to increase uptake of iron through the TfR in erythroleukemic cells and nontransferrin bound iron in HL-60 cells(Chitambar and Sax, 1992), erythroleukemic cells (Perez *et al.*, 2005), and hepatocytes (Sturm *et al.*, 2006). Aluminum and gallium are not essential metals and it is unlikely a mechanism has evolved regulating their levels. Aluminum is particularly important because of its abundance and association with human disease. When aluminum was a component of dialysis tubing, it was shown to causes encephalopathy, anemia, and bone disease. Aluminum is also in drinking water (Allen *et al.*, 1989; Flaten, 2001), several over-the-counter medications, and many vaccines (Yokel and McNamara, 2001), and has been detected in body fluids and tissues (Hershey *et al.*, 1983; Lovell *et al.*, 1993). The association between aluminum and Alzheimer's disease (AD) has been suggested and debated (Yokel *et al.*, 1988).

The major objective of our study is to determine the mechanism by which aluminum increases uptake of nontransferrin bound iron. Glial cells will be studied because of their involvement in iron acquisition in the brain. Astrocytes, for example, express DMT1 (Wang *et al.*, 2002; Wang *et al.*, 2002) and the iron exporter ferroportin (Wu *et al.*, 2004) (Jeong and David, 2003). Astrocytes also express ceruloplasmin, an iron oxidase, which prevents iron mediated oxidative stress (Jeong and David, 2003; Oide *et al.*, 2006). Evidence will be presented demonstrating that relatively low concentrations of aluminum increases uptake of nontransferrin bound iron and uptake of iron bound to transferrin in human glial cells that

requires protein but not RNA synthesis. The amount of iron bound to ferritin decreases, which, overall suggests that aluminum induces responses similar to those observed under iron-deprived condition.

MATERIALS AND METHODS

Cell culture and treatment with aluminum

The U-373 cell line was studied as a model of human astrocytes because it expresses the astroglial marker GFAP (Murphy *et al.*, 1995) astrocyte glutamate transporters (Aronica *et al.*, 2003), and receptors for neurotransmitters such as serotonin (Lieb *et al.*, 2005) and for neuropeptides such as substance P (Bordey *et al.*, 1994). U-373 human glioma cell line was grown in DMEM/10% fetal bovine serum in 100 mm plates. Upon reaching 80% confluency, cells were dislodged with 0.25% trypsin/EDTA and replated at 1:5 split ratio weekly. To examine iron transport, 50×10^4 cells were plated in wells of a 24-well plate and treated with aluminum at 3–4 days after plating.

Aluminum chloride and sodium citrate were made at 1 M fresh daily and were mixed at the appropriate concentrations before adding to DMEM. Cell monolayers were washed thrice with serum-free DMEM and treated with aluminum in DMEM containing 180 uM citriate. Controls received DMEM/citrate. After treatment, cells were washed with DMEM/citrate 5 times before measuring iron uptake.

Uptake of nontransferrin bound iron

A stock solution of ^{55}Fe was made with 10 mM sodium ascorbate that was freshly added to a 1:4 Fe-NTA ($FeCl_2$: nitrilotriacetic acid) to achieve a 20-fold molar excess. To 1 ml of the stock solution, 10 µl of a 10 mCi/ml $^{55}FeCl_3$ was added to give a 1 mM ^{55}Fe solution. Fe-NTA solutions maintain iron solubility and ascorbate maintains iron as Fe^{++}. To initiate dosing, aliquots of the stock solution were added to cells bathed in a uptake buffer containing 1 µM ^{55}Fe in a buffer containing 25 mM HEPES, 1 mM NTA, 150 mM NaCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 37 C unless otherwise indicated. To terminate uptake, cells were placed on ice and washed with ice-cold 10 mM HEPES, 1 mM NTA and 150 mM NaCl. This was repeated 3 times with 5 minute intervals between washings. Cells were lysed with 200 mM NaOH and brought to pH 7.4 with 200 mM HCl and radioactivity was measured by liquid scintillation spectroscopy. Percent control was computed by dividing radioactivity in the presence of aluminum by radioactivity in the control multiplied by 100. Data are presented as percent control with the S.E.M from triplicate cultures.

Uptake of transferrin bound iron

Transferrin laden with iron was prepared by complexing ^{55}Fe to NTA (molar ratio 1:10) and then combining the complex with apotransferrin (molar ratio of 1:4) in 0.5 M Tris buffer/0.1 mM $NaHCO_3$ at pH 8. . Unbound iron was removed by gel filtration on disposable PD10 columns that were developed in DMEM. Transferrin laden with iron at 5 µg/ml was incubated with cells for 60 min in DMEM in the presence of 500 µg/ml apo-transferrin or holo-transferrin. After washing with DMEM, radioactivity was measured by liquid scintillation counting in cell lysates prepared as described above.

Iron binding to ferritin

The transport assay was conducted on cells treated with aluminum and controls as described above except cells were plated in 6-well dishes and incubated with the ^{55}Fe solution for four hours. After washing cells, a lysate was prepared by scraping the cells in PBS and lysing pelleted cells with buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease

inhibitor cocktail from Calbiochem (RIPA). Lysates were concentrated by centrifuging the lysates through YM-100 microfuge filters (Millipore) until the concentrate reached a volume of 10 µl. The concentrates were subjected to native gel electrophoresis in a 7.5% acrylamide gel with a standard horse ferritin. When the standard migrated to approximately one third of the distance of the gel, the gel was removed, dried, and processed for autoradiography.

Western blot analysis of DMT1

Cell monolayers were washed with PBS, and scraped in RIPA buffer, and briefly sonicated. Equal amounts of protein were subjected to SDS-PAGE and Western blotting using an antibody against a N-terminal region on DMT1 as described previously (Cheong *et al.*, 2004). The antibody/antigen complex was detected by the ECL method using reagents from Amersham.

Synthesis of DMT1

Cells were treated overnight with aluminum or media, washed in the transport assay, and incubated for four hours with 10 µCi/ml ^{35}S-methionine (800 with PBS and lysed in buffer containing 50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1% (v/v) Triton X-100 and protease inhibitor cocktail from Calbiochem. Cell lysates were cleared with protein G-agarose (Sigma) and equal amounts of TCA precipitable radioactivity were incubated with 5 µg anti-DMT1 antibody, previously conjugated with 30 µL protein G-agarose. The lysates were incubated overnight at 4°C with rocking. The protein-agarose beads were centrifuged and the pellet was washed three times with cold lysis buffer. The beads were heated to 100 C in SDS-sample buffer and subjected to SDS-PAGE. The gels were dried and processed for autoradiography on Kodak Biomax films.

RESULTS

Aluminum stimulates uptake of iron

U-373 cells stimulated with 15 uM aluminum for 16 hours displayed specific uptake of iron, which was determined by subtracting radioactivity at 4 C. from the radioactivity 37 C. A plateau was reached at 75 uM aluminum, which resulted in approximately 4-fold increase in uptake compared to control cells not treated with aluminum (figure1 A). The dose-response relationship between aluminum and uptake of iron was similar at different pH values. The response at an acidic pH indicates that uptake of nontransferrin bound iron rather than transferrin bound iron is occurring because iron does not bind to transferrin at acidic pH values [Lee, 1998 #4239]. Also, transferrin does not appear involved because uptake is measured in buffer without transferrin.

In addition to temperature dependence, another characteristic of a transporter is that uptake reaches saturation. In the glial cell line, the uptake of iron reached saturation at concentrations greater than 25 µM in cells stimulated with aluminum (figure 1 B). Nonlinear regression analysis computed a Km of 6.9 uM and Vmax of 1.59 fmol/min/ug protein. Overall, it appears that aluminum stimulates uptake of nontransferrin bound iron through a transporter.

We also examined the human intestinal cell line Caco-2 cells because of the importance of the intestine in maintaining iron homeostasis. Iron uptake was observed at 80 uM aluminum and was also not dependent on pH (figure 1 C), which indicates that aluminum stimulates uptake of nontransferrin bound in iron in human intestinal cells.

Citrate attenuates the response to aluminum

Aluminum forms a complex with citrate in serum and cerebral spinal fluid. In endothelial cells, for example, the uptake of aluminum is mediated, in part, by the citrate transporter (Yokel, 2000). The response of glial cells to aluminum, however, did not appear to require the citrate transporter (figure 2 A). A rather specific inhibitor of the transporter, cyano-hydroxycinnamic acid (CHCA), nor a less specific inhibitor, 4,4'-diisosthiocyanodihydrostibene-2,2'-disulphonic acid (DIDS), affected the response to aluminum. Interestingly, the response was higher when citrate was not included in the media buffer (figure 2 B) and the response to aluminum was lost when the citrate concentration was raised to 1 mM (data not shown).

Other metals stimulate uptake of nontransferrin bound iron

Other metals were examined that are commonly found in the environment or have been suggested to effect iron homeostasis. Iron uptake was stimulated by ferrous ammonium citrate and tin (figure 3). Several other divalent metals were not effective.

Length of time needed for aluminum to simulate uptake of iron

The dependence on temperature, and that iron uptake reached saturation, suggests that aluminum is stimulating uptake of nontransferrin bound iron by activating a transporter. Activation could be achieved directly by physically changing the transporter or a co-transporter, or aluminum might be increasing the expression of a transporter. We reasoned that a direct interaction would be observed very quickly and would not require the 16-hour incubation that has been used. In examining nontransferrin bound iron uptake in cells treated with aluminum for different lengths of time, 60 minutes was the shortest time interval in which a significant increase was observed (figure 4 A). Maximum transport was observed at approximately after a 2 hour incubation with aluminum.

The decay in aluminum-stimulated transport was examined after washing the cells free from aluminum. Higher transport of nontransferrin bound iron was maintained for 4 hours and was almost completely lost at 6 hours (figure 4 B).

Aluminum-stimulated uptake of nontransferrin bound iron requires protein not RNA synthesis, and glycosylation

One possible explanation for the minimum 6 0-minute time interval is that aluminum stimulates nontransferrin bound iron transport by increasing the expression of a transporter. To increase expression, RNA and protein or only protein synthesis should be involved. Interestingly, the protein synthesis inhibitors anisomycin and puromycin blocked stimulation by aluminum but the RNA synthesis inhibitor actinomycin D was ineffective (figure 5). RNA and protein synthesis inhibitors were used at concentrations that blocked macromolecule synthesis between 60–75% (data not shown) Protein synthesis and RNA synthesis were determined by measuring ^{3}H-leucine and ^{3}H-uridine uptake, respectively, into trichloroacetic acid precipitates. Tunicamycin also inhibited aluminum stimulated uptake of nontransferrin bound iron, which suggests the involvement of glycosylation.

Aluminum treatment does not affect levels of DMT1

The data so far suggests that aluminum is stimulating uptake of nontransferrin bound iron by increasing the expression of a transporter. The effects of aluminum on DMT1 were examined because it is expressed on astroglial endfeet in vivo (Wang *et al.*, 2001). Western blot analysis, however, revealed no change in levels of DMT1 in cells treated overnight with 100 μM aluminum (figure 6 A). DMT1 displayed a molecular weight of approximately 60,000, which is similar to other studies on glial cells (Lis *et al.*, 2004).

Small changes in synthesis might not be observed in Western blots. To more carefully examine whether aluminum affects synthesis, the amount off ^{35}S-methionine incorporated into DMT1 was measured. Similar to the observations in the Western blot analysis, aluminum did not affect the synthesis of DMT1. Two bands were observed within the molecular weight range reported for DMT1 (figure 6 B). The differences in molecular weight are likely due to differences in glycosylation.

Aluminum stimulates uptake of iron through the TfR

Aluminum might affect other mechanisms involved in iron homeostasis, for example TfR and ferritin. To examine TfR-dependent iron uptake, transferrin laden with ^{55} Fe was incubated in cells treated overnight with aluminum. Increased uptake of iron was observed in cells treated with 40 and 100 μM aluminum (figure 7 a). To verify that the iron was taken up through the TfR, transferrin bound iron was competed with 100x holo-transferrin and apo-transferrin. Only holo-transferrin was found to compete. Similar to uptake of nontransferrion bound iron, puromycin inhibited the effects of aluminum on iron uptake through the TfR (figure 7 B).

Less iron is bound to ferritin after aluminum simulation

The effect of aluminum on ferritin levels was also examined. After incubating cells with ^{55}Fe for four hours, ferritin was isolated from the cytosol by ultrafiltration through a filter with a 100,000 mw cutoff and subjected to native gel electrophoresis. Control cells displayed a denser ferritin band than the cells treated with aluminum even though cells treated with aluminum contained approximately almost 4-fold more total radioactivity (figure 8).

DISCUSSION

The results presented here demonstrate that aluminum at low concentrations strongly stimulates uptake of nontransferrin bound iron in a human glial cell line. The evidence supporting that transferrin was not involved is that uptake was conducted in the absence of transferrin and that uptake was similar at acidic and neutral pH values. Acidic conditions would have resulted in the release of iron from transferrin (Lee and Goodfellow, 1998). A transporter appears to mediate the uptake of nontransferrin bound iron because uptake reached saturation and was temperature-dependent. The affinity of the putative transporter for nontransferrin bound iron was in the low micromolar range, which is similar to affinities of other cell surface metal transporters including DMT1 (Bannon *et al.*, 2002) and zinc transporters (Gaither and Eide, 2000).

We were unable to identify the transporter. DMT1 was a possible candidate because it has been suggested to mediate iron acquisition in astrocytes (Wang *et al.*, 2001; Wang *et al.*, 2002). Astrocytes do not express TfR in vivo (Moos, 1996; Moos and Morgan, 2002). Acidic conditions should have stimulated transport because DMT1 is proton coupled (Gunshin *et al.*, 1997) but they did not. Additionally, protein synthesis was required for the effects of aluminum but changes in levels of DMT1 were not observed in Western blots nor in the synthesis of DMT1. Thus, one explanation for our results is activation of a different transporter for nontransferrin bound iron. Recent identified candidates include zip14 (Liuzzi *et al.*, 2006) and lipocalins (Yang *et al.*, 2002). It is also possible, however, that aluminum increases levels of auxillary proteins working with DMT1. An auxillary protein might mediate the translocation DMT1 to the cell surface or provide protons to DMT1 at physiological pH thereby creating an optimum microenvironment for transport. Another reason for an auxillary protein is to prevent iron-mediated oxidative stress. DMT1 transports the ferrous form of iron, which would catalyze oxygen radicals at the cell surface or inside

the cell. Likely a mechanism evolved preventing the generation of these radicals in DMT1-mediated iron transport. One possibility is that iron is in vesicles and shielded from targets of oxidative stress. GTP-binding proteins are involved in forming vesicle and associate with DMT1 in Caco-2 cell (Moriya and Linder, 2006) and neurons (Cheah *et al.*, 2006).

Our study is now one of several that have shown increases in uptake of nontransferrin bound iron and transferrin bound iron in cells treated with aluminum [Sturm, 2006 #4258][Perez, 2005 #4259][Abreo, 1999 #4305]. Additionally, we observed decreases in the amount of iron bound to ferritin though the uptake of iron was four-fold higher in cells treated with aluminum. The cellular responses to aluminum and iron deficiency are similar because both involve increases in uptake of iron through the TfR and decreases in ferritin. Aluminum might induce a “pseudo iron deficient status” because it has been shown to stabilize interactions between IRP2 and the IRE (Yamanaka *et al.*, 1999). Aluminum did not, however, affect levels of DMT1. There are two possible explanations. First, DMT1 mRNA might be less responsive to the effects of aluminum. Two forms of DMT1 mRNA have one IRE on the 3’ untranslated region but TfR mRNA has five. Indeed, a 20 h treatment with iron chelators increased expression of the TfR mRNA but not the DMT1 mRNA in a fibroblast cell line (Wardrop and Richardson, 1999). Another possibility is that U-373 cells predominantly express the DMT1 mRNA forms missing the IRE.

Although aluminum has the same effects on TfR and ferritin as iron status, the distinct difference is that the response to iron deprivation is an adaptive response and the iron is utilized for different functions such as respiration. Conversely, aluminum is a xenobiotic and adaptive responses have likely not evolved. Consequently, the iron taken up by cells in response to aluminum is not needed. The levels of ferritin are low, which might result in higher levels of oxidative stress due to more free iron. We suggest that the effects of aluminum on iron homeostasis might explain recent observations demonstrating increases in oxidative stress and inflammation in vitro and in vivo after exposure to aluminum (Campbell *et al.*, 2004; Schuhmacher *et al.*, 2004; Jyoti and Sharma, 2006).

The interest in aluminum was initiated from studies demonstrating the neurotoxicity of aluminum and the observation of AD-like pathology in patients who were exposed to aluminum through kidney dialysis (Alfrey *et al.*, 1976) (Harrington *et al.*, 1994). The lowest effective concentration of aluminum found here, 15 μM, is approximately twice the serum concentration reported to induce classical dialysis induced encephalopathy that was reported in earlier studies (Flaten *et al.*, 1996) though levels have dropped because of changes in dialysis tubing and medications. More recent studies, however, have suggested an association between the occurrence of AD and aluminum at much lower serum levels through exposure in the drinking water (Flaten, 2001). Also, autopsy studies demonstrated higher levels of aluminum in AD brains (Bouras *et al.*, 1997; Walton, 2006) and the induction of a disease similar to AD in rabbits exposed to aluminum (Yokel *et al.*, 1988) though other studies have failed to find associations (Flaten, 2001) and have not detected aluminum in AD brains (Lovell *et al.*, 1993; Makjanic *et al.*, 1998; Reusche *et al.*, 2001). AD is a complex disease and sporadic cases of AD likely have different etiologies and are associated with mixtures of various environmental pollutants. We suggest that the commonality among these pollutants is their interactions with mechanisms regulating metal homeostasis. The consequences of disrupting transporters and other proteins regulating metal homeostasis could be higher levels of metals, which have been detected in AD brains. Indeed, zinc transporter knockout mice (Lee *et al.*, 2002; Friedlich *et al.*, 2004) and mice with a mutant ATPase7b copper transporter (Phinney *et al.*, 2003) fail to develop plaques when overexpressing the human amyloid precursor protein. The inconsistencies in detecting aluminum in AD brains might simply reflect the different mechanisms in which metal transporters are regulated and their sensitivity to different environmental pollutants.

In summary, our observations indicate that aluminum increases uptake of transferrin bound iron and the transport of nontransferrin bound iron. Aluminum increases uptake of nontransferrin bound iron by increasing the expression of an unidentified transporter or a protein that interacts with DMT1. Because levels of ferritin decrease, the higher levels of intracellular iron are possibly unbound and might be available to increase oxidative damage.

Acknowledgments

The research was supported by the Gerber Foundation and the National Institutes of Health Center Grant (03819).

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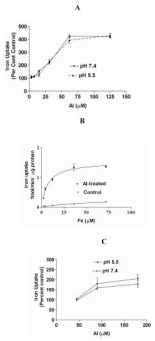

Figure 1. The effects of aluminum on uptake of iron on human glial and intestinal cells

U-373 cells were treated for 16 h with different concentrations of aluminum chloride in DMEM/180 uM sodium citrate. After washing cells with DMEM/citrate, uptake was initiated by adding 1μM ^{55}Fe in uptake buffer with 25 mM HEPES at pH 7.4 or 25 mM MOPS at pH 5.5 (A). Michaelis-Menten kinetics (B) for iron uptake was measured at pH 7.4 by incubating cells with different concentrations of iron and determining specific uptake by subtracting uptake at 4 °C (non-specific uptake) from total uptake at 37 C. The effects of aluminum on uptake of iron were also examined in the human intestinal cell line Caco-2 cells (C). Data are reported as the per cent control (A, C) and expressed as means ± S.E.M. from triplicate cultures.

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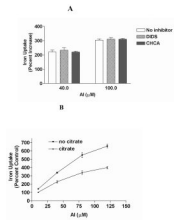


Figure 2. The involvement of citrate transporter on the response to aluminum

U-373 cells were incubated with 2 mM cyano-hydroxycinnamic acid (CHCA), 100 μM 4,4'-diisothiocyanodihydrostilbene-2,2'-disulphonic acid (DIDS), or alone, in the presence or absence of aluminum at 40 and 100 μM. The percent control was computed using the citrate inhibitor without aluminum as the control. An aluminum dose-response curve was examined in the presence (180 μM) and absence of citrate (B). After washing aluminum from cells, iron uptake was measured as described in figure 1 and Materials and Methods. Data are reported as the per cent control and expressed as means ± S.E.M. from triplicate cultures.

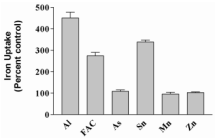
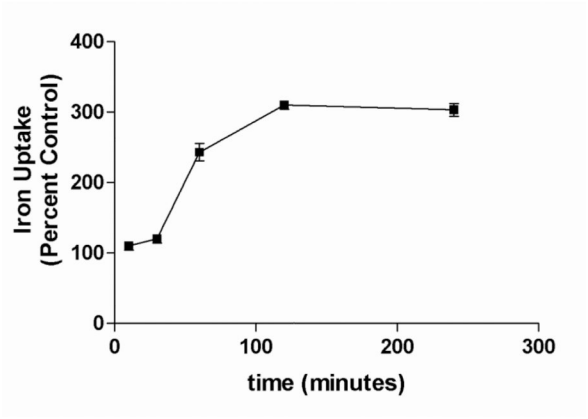


Figure 3. The effect of different metals on iron uptake

Cells were incubated in the presence of 100 uM concentrations of aluminum chloride, ferrous ammonium citrate, arsenic, tin chloride, manganese chloride, and zinc chloride in DMEM with 180 uM citrate overnight. Controls were not incubated with metals. Data are reported as the per cent control and expressed as means ± S.E.M. from triplicate cultures.

A



B

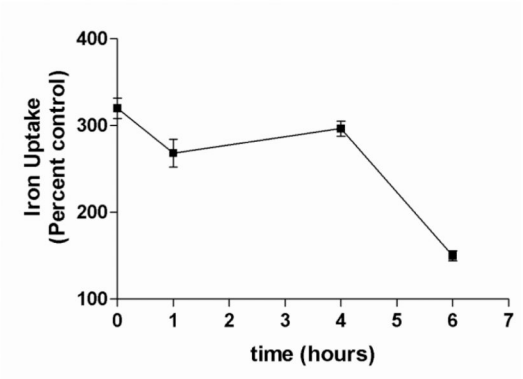


Figure 4. The relation between length of time and iron uptake

The uptake of iron was measured in U-373 cells that were incubated with 120 uM for 2, 30, 60, 120, and 240 minutes (A). In B, cells were incubated with 120 uM aluminum chloride overnight and washed with media without aluminum containing 180 uM sodium citrate. Controls were treated similarly but without aluminum. Accordingly, each time point has a separate control. After different lengths of time, uptake of iron was measured. Data are reported as the per cent control and expressed a means ± S.E.M. from triplicate cultures.

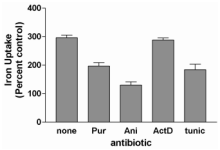


Figure 5.

The effect of inhibitors of protein and RNA synthesis and glycosylation on aluminum-mediated stimulation of iron uptake. U-373 cells were treated for 16 hours with or without 100 uM ammonium chloride in DMEM/ citrate and 1 μg/ml puromycin, 1 ug/ml anisomycin (Ani), 2 μg/ml actinomycin D (ActD), or 2 μg/ml tunicamycin (tunic). Data are reported as per cent control (radioactivity from cultures treated with aluminum and inhibitor was divided by radioactivity in cultures treated with the same inhibitor x 100) and expressed as means ± S.E.M. from triplicate cultures.

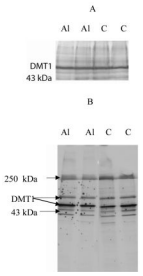


Figure 6.

The effect of aluminum on DMT1. To measure DMT1 in Western blots, lysates were prepared from cells treated with 100 μM aluminum or remain untreated (C) and subjected to SDS-PAGE and Western blotting. An antibody against N-terminal sequence (amino acids 1–41) was used in the blots as previously reported (Cheong *et al.*, 2004) and antibody binding was detected by ECL (A). Similarly, the antibody was used to immunoprecipitate DMT1 from U-373 cells treated with 100 μM aluminum overnight and incubated with 35S-methionine for four hours. DMT1 was immunoprecipitated as described in the text (B).

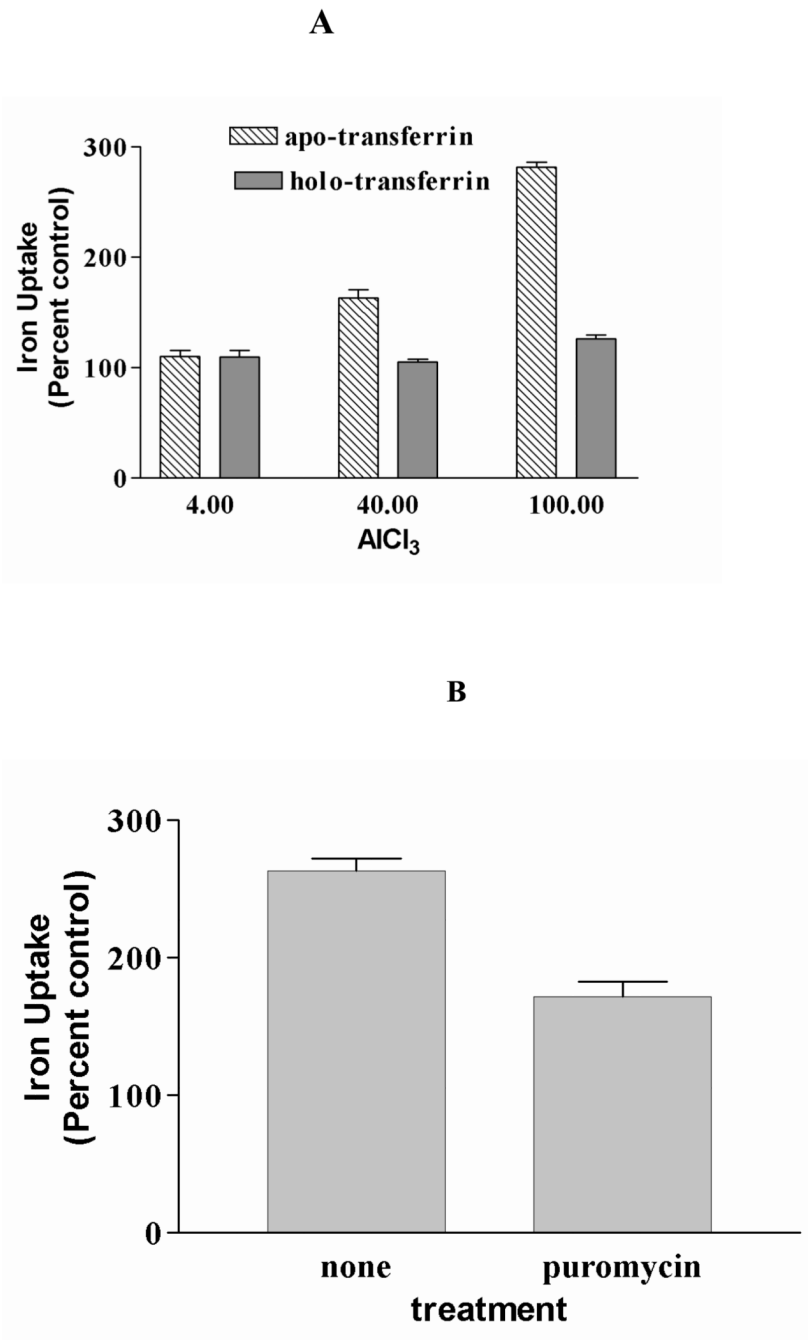


Figure 7.

Uptake of transferring bound iron in cells treated with aluminum. Cells were incubated with transferrin bound with ^{55}Fe for 60 minutes after treatment with 100 uM aluminum. Uptake of transferrin was examined in the presence of a 100 x competitor (holo-transferrin or apo-transferrin) (A). The effect of puromycin on uptake was also examined as described in figure 6 without competitor (B). Data are reported as the per cent control and expressed as means ± S.E.M. from triplicate cultures.

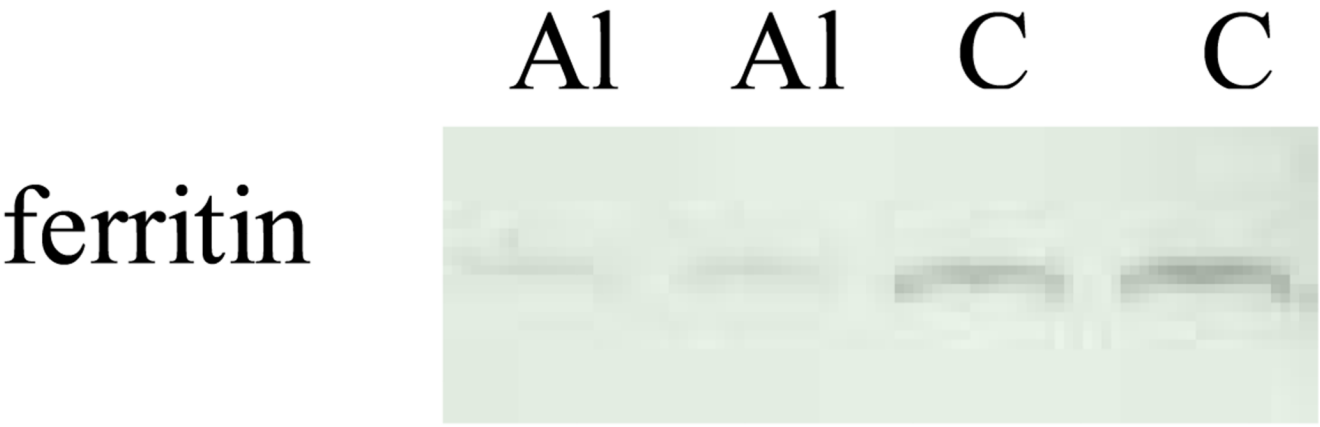


Figure 8.

The effect of aluminum on ferritin. To measure the amount of iron bound to ferritin, cells were treated with 100 μM aluminum chloride (Al) in DMEM/180 uM sodium citrate or without aluminum (C), washed with DMEM/180 uM citrate, and incubated with ^{55}Fe for fours at 37 °C. ^{55}Fe levels were 4.3 fold greater in cells treated with aluminum compared to controls (data not shown). Ferritin was isolated and subjected to native gel electrophoresis as described in the text. An autoradiogram is shown.