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Zinc retention differs between primary and transformed cells in response to zinc deprivation

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Abstract

Previous studies in our laboratory have demonstrated that reducing the availability of zinc with the extracellular metal chelator DTPA (diethylenetriaminepentaacetate) enhances, rather than inhibits, the thyroid hormone induction of growth hormone mRNA in GH3 rat anterior pituitary tumor cells. To understand the actions of the chelator on cellular zinc status, we observed the effects of DTPA on ^{65}Zn uptake and retention. DTPA reduced the uptake of ^{65}Zn by GH3 cells from the medium, but when GH3 cells were prelabeled with ^{65}Zn, it resulted in greater retention of the isotope. In primary hepatocytes, DTPA both reduced the uptake of ^{65}Zn from the medium and increased efflux from prelabeled cells. To investigate this difference, we studied the effects of DTPA on radioactive zinc flux in the H4IIE (rat hepatoma), MCF-7 (human breast cancer) and Hs578Bst (nontransformed human mammary) cell lines and in rat primary anterior pituitary cells. DTPA reduced the uptake of ^{65}Zn in all cell lines examined. DTPA increased the retention of ^{65}Zn in prelabeled H4IIE, MCF-7 and Hs578Bst cells but reduced it in primary pituitary cells. Time course experiments showed that ^{65}Zn efflux is shut down rapidly by DTPA in transformed cells, whereas the chelator causes greater efflux from primary hepatocytes over the first 6 h. Experiments with ^{14}C-labeled DTPA confirmed that this chelator does not cross cell membranes, showing that it operates entirely within the medium. Expression of ZnT-1, the efflux transporter, was not affected by DTPA in H4IIE cells. Thus, zinc deprivation enhanced zinc retention in established cell lines but increased efflux from primary cells, perhaps reflecting differing requirements for this mineral.

Keywords: Zinc; Zinc uptake; Zinc retention; ^{65}Zn; Cultured cells; Cancer

1. Introduction

Zinc is an important micronutrient required for many basic cellular processes, including transcription and progression through the cell cycle [1,2]. It is an essential component in more than 300 enzymes and forms part of an even larger number of transcription factors [3]. This necessitates a finely regulated mechanism for the maintenance of zinc concentrations inside cells. It has been shown that tissue and cellular zinc concentrations can remain unchanged even in conditions of zinc deficiency [4]. Studies done with 3T3 cells have shown that in conditions of severe zinc deficiency caused by the extracellular metal chelator DTPA (diethylenetriaminepentaacetate), although DNA synthesis was suppressed, total cellular zinc concentrations remain unchanged [5]. This implies that only a fraction of the zinc in the cell is affected. The free zinc concentrations that affect the transcription of the zinc regulatory machinery are in the femtomolar range, suggesting that cytosolic concentrations of free zinc inside the cells are tightly controlled [6]. These studies are indicative of a number of regulatory mechanisms that work in concert to prioritize the distribution of zinc to various cellular processes.

The tissue concentrations of zinc have been measured in various cancer tissues and compared with surrounding normal tissues. Zinc concentrations of cervical cancer and uterine myeloma were found to be significantly lower than the surrounding nonlesion tissues [7]. A similar observation was seen with lung cancer [8]. However, it has been reported

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that zinc concentrations in mammary tumors are elevated in comparison with normal mammary tissues [9,10]. Furthermore, it has been shown that labile intracellular zinc is increased in proliferating 3T3 cells and that this could be due to an increase in the retention of extracellular zinc [11]. These studies indicated that zinc concentrations of malignant and nonmalignant tissues vary significantly, suggesting a difference in their homeostatic mechanisms.

Previous work in our laboratory has shown that chelation of zinc by DTPA enhances, rather than inhibits, the induction of growth hormone by T3 in GH3 rat anterior pituitary tumor cells [12]. This effect was reversed by the addition of zinc. EDTA, another chelator, also induced GH expression in the presence of thyroid hormone. EDTA was less potent than DTPA, in keeping with their affinities for zinc [13]. These results indicated that perhaps DTPA affects zinc homeostasis in GH3 cells in a manner different from its predicted role of simply restricting zinc entry or pulling zinc out of the cells. Hence, to further investigate the effects of DTPA, the cellular uptake and redistribution of ^{65}Zn were studied in both tumor cell lines and freshly isolated primary cells. Here, we tested the hypothesis that zinc exchange with the medium differs between primary and transformed cultured cells from the same tissue of origin under conditions of zinc deprivation. In addition, we compared ^{65}Zn flux in two established human breast cell lines, one derived from a tumor and another from normal tissue.

2. Materials and methods

2.1. Culture of cell lines

GH3 rat anterior pituitary tumor cells, H4IIE rat hepatoma cells, MCF-7 human breast cancer cells and Hs578Bst normal human epithelial breast cells were obtained from ATCC (Manassas, VA). GH3 cells were maintained in Ham's F-10 medium supplemented with 2.5% newborn calf serum and 12.5% horse serum in the presence of 0.1 mM nonessential amino acids and 1 mM Na pyruvate. H4IIE, MCF-7 and Hs578Bst cells were maintained in minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum, nonessential amino acids (0.1 mM) and Na pyruvate (1 mM). MCF-7 cells were also supplemented with bovine insulin (10 mg/L); Hs578Bst cells, with 30 μg/L of epidermal growth factor and $NaHCO_3$ (1.5 g/L). Penicillin (50,000 IU/L) and streptomycin (50 mg/L) were added to all media. Cells were kept in a humidified CO_2 incubator (37°C, 5% CO_2). For experiments, 5×10^6 cells were subcultured into 25-cm^2 flasks in a 5-ml medium and then used at about 90% confluency. Medium components were obtained from Sigma (St. Louis, MO).

2.2. Isolation and culture of rat hepatocytes

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 200 and 300 g were anaesthetized using 5% isoflurane (Henry Schein, Melville, NY) in oxygen using a vaporizer. The peritoneal cavity was exposed quickly and a ligature was secured around the inferior vena cava, above the kidney, to isolate the delivery of perfusate to the liver. A cannula was introduced into the superior vena cava through the right atrium, and the liver was cleared of blood by perfusing warmed EGTA Tricine buffer (0.025 M Tricine, 4 mM KCl, 0.44 mM KH_2PO_4, 0.137 M NaCl, 0.65 nM Na_2HPO_4 and 0.4 mM EGTA, pH 7.6) at a rate of 25 ml/min for about 5 min. The liver was digested by perfusing type I collagenase (100 mg, Worthington Biochemical, Lakewood, NJ) dissolved in 250 ml of Hank's buffered salt solution (HBSS; Sigma) at a rate of 20 ml/min for approximately 10 min until the liver connective tissue visibly began to disintegrate. The liver was teased apart in MEM to disaggregate the cells, the tissue debris was allowed to settle out and cells were collected by centrifugation at 250×*g* for 5 min. The cell pellet was resuspended in 30 ml of modified William's E medium containing L-glutamine and Na pyruvate (Invitrogen, Grand Island, NY) supplemented with sodium bicarbonate (26 mM), Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 23 mM], dexamethasone (10 nM), human recombinant insulin (1 nM, Invitrogen), penicillin (50,000 IU/L) and streptomycin (50 mg/L), pH 7.2. The cells were counted using a hematocytometer and plated on 10-cm Primaria tissue culture plates (Benton Dickinson, Franklin Lakes, NJ) to a density of 4×10^6 cells/plate. Cells were allowed to attach for 4 h. The medium was removed, and 10 ml of fresh medium was added to each plate, with the appropriate treatment. All animal procedures were approved by the University of Connecticut Animal Care and Use Committee.

2.3. Isolation and culture of rat anterior pituitary cells

The anterior pituitary was dissected and cells were cultured as described by Lamberts et al. [14], with slight modifications. Following CO_2 euthanasia of male Sprague–Dawley rats, the skull was opened to reveal the pituitary gland, which was carefully lifted off the base of the skull. The glands were placed in a Petri dish containing HBSS, and the anterior lobes were separated out under a dissecting microscope. The anterior lobes were rinsed with HBSS, sliced using a sterile blade and washed twice with HBSS to remove blood and connective tissue. They were incubated with dispase solution (2500 U/L) (Roche, Nutley, NJ) on an orbital shaker for 2 h at 37°C. The cells were collected by centrifugation at 400×*g* for 5 min, and the cell pellet was washed with HBSS. The cell pellet was resuspended in MEM supplemented with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (50,000 IU/L), streptomycin (50 mg/L) and 10% fetal calf serum. The cells were resuspended using a Teflon glass homogenizer. The heavy cellular debris was allowed to settle down, and the supernatant with the cells was collected. The cells were counted using a hematocytometer, seeded into six-well plates at a final concentration of 1.3×10^5 cells/well and incubated in a humidified CO_2 incubator overnight.

2.4. Cellular uptake of ^{65}Zn

Radioactive ^{65}Zn ($ZnCl_2$; 62.5 MBq/mg) was purchased from Perkin Elmer (Bilerica, MA). Cells were incubated with 500 Bq of ^{65}Zn in the presence or absence of 50 μM DTPA for 48 h. The medium was collected; the cells were rinsed with HBSS, detached with trypsin-EDTA and collected by centrifugation at 250×*g*. The cell pellets were then homogenized in STMT buffer (0.25 M sucrose/20 mM Tris–HCl/1.1 mM $MgCl_2$/1% Triton X100, pH 7.8) with five strokes of a Teflon glass homogenizer. The homogenates were centrifuged at 1500×*g* for 10 min to pellet the crude nuclear fraction. The ^{65}Zn content of the medium, cell supernatants and crude nuclear pellets was measured by gamma spectroscopy (Cobra II System, Packard, Meriden, CT).

2.5. Cellular retention of zinc

Cultured cells were incubated with 1000 Bq of ^{65}Zn for 48 h. The radioactive medium was discarded; cell monolayers were rinsed with HBSS and then incubated with fresh medium in the presence or absence of 50 μM DTPA for a further 48 h. ^{65}Zn content of cell supernatants, crude nuclear pellets and medium was measured as before.

2.6. Time course of ^{65}Zn efflux from cells in the presence of DTPA

Cultured cells were prelabeled with 1000 Bq of ^{65}Zn for 48 h, the medium was discarded and the cells were rinsed with HBSS. Cultures were incubated in fresh medium in the presence or absence of 50 μM DTPA for various times from 30 min to 6 h. At the end of the incubation period, the medium was collected, cells were rinsed with HBSS and detached and the ^{65}Zn content of medium and cells was determined by gamma spectroscopy.

2.7. Membrane impermeability of DTPA

Experiments were performed with both GH3 cells and primary hepatocytes to confirm the membrane-impermeable nature of DTPA. Monolayers were incubated with 100 Bq/plate of ^{14}C-labeled DTPA (specific activity=1.9 GBq/mmol; Amersham, Piscataway, NJ) for 48 h. The medium was collected, cells were rinsed with HBSS and detached with trypsin and cellular and crude nuclear fractions were prepared as described previously. The nuclear pellet was dissolved in 100 μl of 0.1 M NaOH and transferred to a scintillation vial. Medium and cell fractions were counted in 10 ml of ScintiSafe Plus 50% (Fisher Scientific) in a Beckman model LS 6500 scintillation counter.

2.8. Expression of ZnT-1 mRNA and protein in H4IIE cells

H4IIE cells were treated with or without 50 μM DTPA for 48 h. Total RNA was extracted from the cells using Trizol reagent (Invitrogen). ZnT-1 mRNA was quantified using semiquantitative RT-PCR (OneStep RT-PCR Kit, Qiagen, Valencia, CA). The primers for ZnT-1 (5′-GTTTTCCTGATCCCTGCAA and 3′-CCTACGCTAGC-GAATTCAGG) and glyceraldehyde phosphate dehydrogenase (GAPDH; 5′-CATGCCAGTGAGCTTCCCGTT and 3′-GTGGAGTCTACTGGCGTCTTC) were designed using Primer 3 software (http://frodo.wi.mit.edu/). Amplification was carried out at an annealing temperature of 58°C for 35 cycles using a GeneAmp PCR System 9700 (Applied Biosystems, CA) thermal cycler. The amplified DNA was visualized by ethidium bromide staining following agarose gel electrophoresis.

Proteins were isolated using ice-cold RIPA lysis buffer (Teknova, Hollister, CA) with protease inhibitor cocktail (Sigma), and concentrations were determined with a Bio-Rad DC Assay Kit (Bio-Rad Laboratories, Hercules, CA). The protein samples (20 μg) were separated by SDS-PAGE and electrotransferred to nitrocellulose membrane (Pierce, Rockford, IL). After blocking for 1 h (0.5% nonfat dry milk in TBS/T [0.1% Tween-20 in Tris-buffered saline]), the membranes were washed thrice for 5 min with TBS/T and incubated overnight with anti-ZnT-1 (generously provided by Drs. J. Luizzi and R.J. Cousins, University of Florida) and anti-actin (Sigma) primary antibodies in blocking buffer. The membrane was rinsed thrice for 5 min each with TBS/T and incubated with horseradish peroxidase-coupled secondary antibody (Sigma) for 1 h and washed 3×5 min with TBS/T before developing using an enhanced chemiluminescence system (Pierce).

2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA; SPSS version 13.00) using Tukey's test for post hoc analysis. Time course studies were analyzed using two-way ANOVA with Bonferroni post hoc analysis. Each treatment consisted of three to six replicates, and each experiment was performed at least twice.

3. Results

The membrane impermeability of DTPA was determined by using its ^{14}C-labeled form in GH3 cells and primary hepatocytes. Greater than 99% of added radioactivity was recovered from the extracellular medium, demonstrating that DTPA is a plasma membrane-impermeable compound (Table 1).

Initial studies examined the effects of DTPA on uptake and retention of ^{65}Zn by GH3 cells. Addition of DTPA to the medium reduced the uptake of label by these cells to about 10% of control cells (Fig. 1A). Uptake of ^{65}Zn by the crude nuclear pellet was coordinately reduced such that it retained a constant proportion of cellular radioactivity. However, when GH3 cells were prelabeled with ^{65}Zn, DTPA proved to be ineffective for eliciting zinc efflux. In fact, cells incubated with DTPA retained about a third more of their initial content of ^{65}Zn than cells incubated in its absence (Fig. 1B). Again, DTPA proportionately

Table 1
Data supporting the membrane impermeability of DTPA

	Recovered radioactivity (%)	
	GH3 cells	Primary hepatocytes
Medium	99.15±0.19	99.86±0.57
Trypsin wash	0.76±0.20	0.12±0.02
Total cell	0.09±0.03	0.02±0.00
Nucleus	0.001±0.007	0

Cells were incubated in medium containing ^{14}C-labeled DTPA for 48 h. The medium was removed, and the cells were rinsed with HBSS, detached with trypsin and homogenized to separate the nuclear fraction. Radioactivity in each fraction was quantified by liquid scintillation counting. Values represent the mean±S.D. of five replicates, and each experiment was repeated once.

affected crude nuclear ^{65}Zn such that it retained an unchanging fraction of cellular radioactivity.

Similar experiments were then performed with primary rat hepatocytes. When added concurrently to the medium with the label, DTPA effectively reduced the amount of ^{65}Zn taken up by the cell by about 85% (Fig. 2A). In this case, it also reduced the fraction of cellular radioactivity associated with the crude nuclear pellet, demonstrating a disproportionate effect on this fraction. DTPA also proved to be effective at inducing efflux of ^{65}Zn from prelabeled primary hepatocytes. DTPA reduced cellular retention of ^{65}Zn to about a third of that seen in its absence (Fig. 2B). It also caused greater efflux of ^{65}Zn from the crude nuclear fraction such that it accounted for only about 18% of total cellular radioactivity in the presence of DTPA, compared with 37% of the greater total in its absence.

There are many differences between GH3 rat pituitary tumor cells and primary hepatocytes that might underlie the altered response to DTPA with respect to cellular ^{65}Zn retention and the proportion of radioactivity associated with the crude nuclear fraction. To determine which of these differences might be responsible, we next examined the

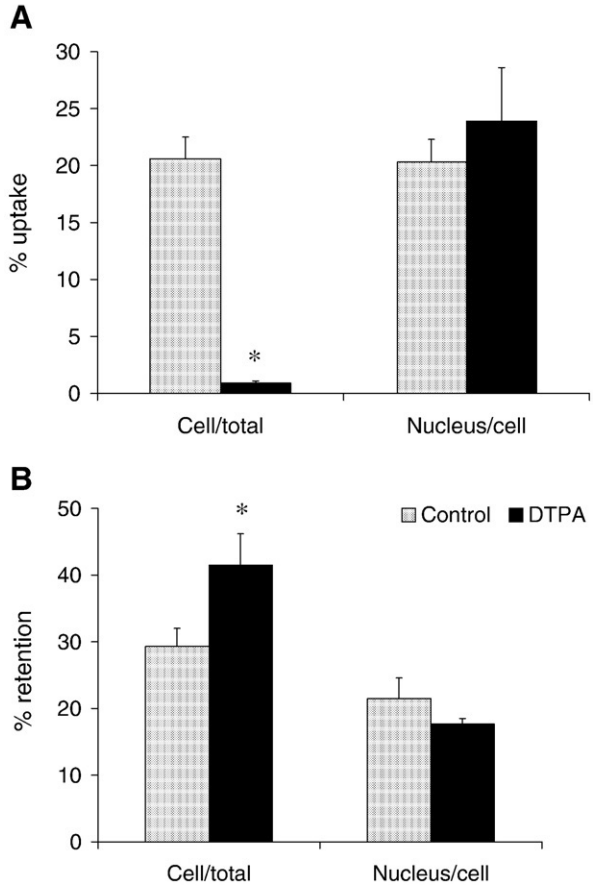

Fig. 1. Effect of DTPA on uptake (A) and retention (B) of ^{65}Zn in GH3 cells. Cells were incubated with ^{65}Zn with or without DTPA (50 μM) for 48 h to measure zinc uptake. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. "Cell/total" represents cellular ^{65}Zn expressed as a percentage of the total recovered in all fractions. "Nucleus/cell" represents crude nuclear ^{65}Zn expressed as a percentage of the total cellular radioactivity. Values are the mean±S.D. of five experiments, each including at least three replicates of each treatment. The asterisk indicates significant difference from control, $P<.001$.

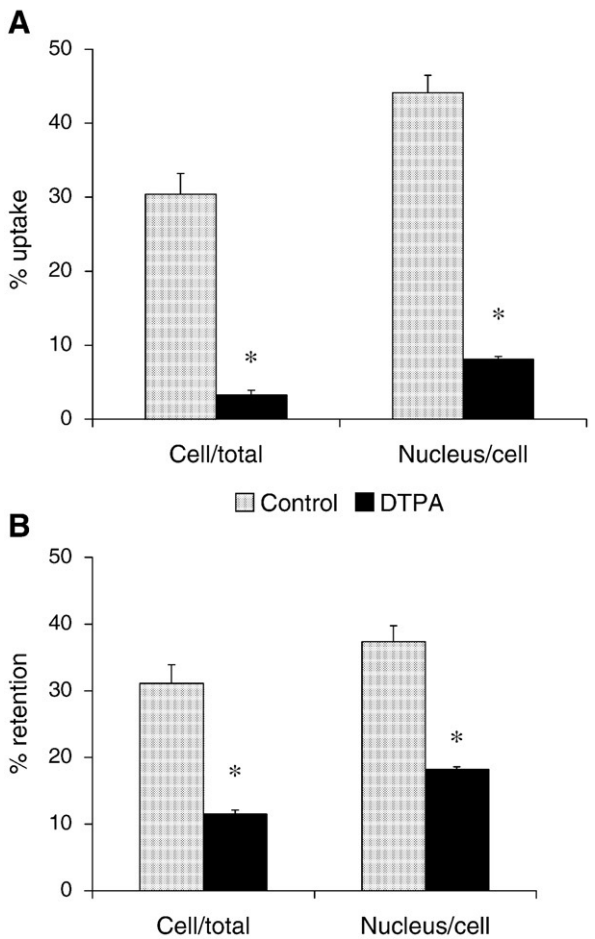

Fig. 2. Effect of DTPA on uptake (A) and retention (B) of ^{65}Zn in rat primary hepatocytes. Cells were incubated with ^{65}Zn with or without DTPA (50 μM) for 48 h to measure zinc uptake. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. Values are the mean±S.D. of three experiments, each including at least three replicates of each treatment. The asterisk indicates significant difference from control, $P<.001$.

effects of DTPA on zinc flux in H4IIE rat hepatoma cells. H4IIE cells share a tissue of origin with primary hepatocytes but, like GH3 cells, are a tumor cell line. DTPA reduced the uptake of ^{65}Zn by H4IIE cells by about 50% (Fig. 3A). However, it did not affect the proportion of that radioactivity associated with the nucleus. DTPA treatment resulted in greater retention of ^{65}Zn in prelabeled H4IIE cells (Fig. 3B). Retention of ^{65}Zn was greater than 60% in the presence of 50 μM DTPA, compared with 40% in its absence. DTPA also resulted in greater retention of ^{65}Zn in the crude nuclear pellet such that it retained a constant proportion of cellular radioactivity (~20%). Overall, it appeared that the rat hepatoma cells responded to DTPA in a manner similar to the rat pituitary tumor cells and different from the primary hepatocytes.

The time course of efflux of ^{65}Zn in the presence and that in the absence of DTPA were next compared across these sets of cultured cells to determine how early after addition of DTPA would differences in response become apparent. Cells were prelabeled with ^{65}Zn and incubated in the presence or absence of 50 μM DTPA; radioactivity was then quantified in cells and medium over the succeeding hours. In all three cell types incubated without DTPA, cellular radioactivity was

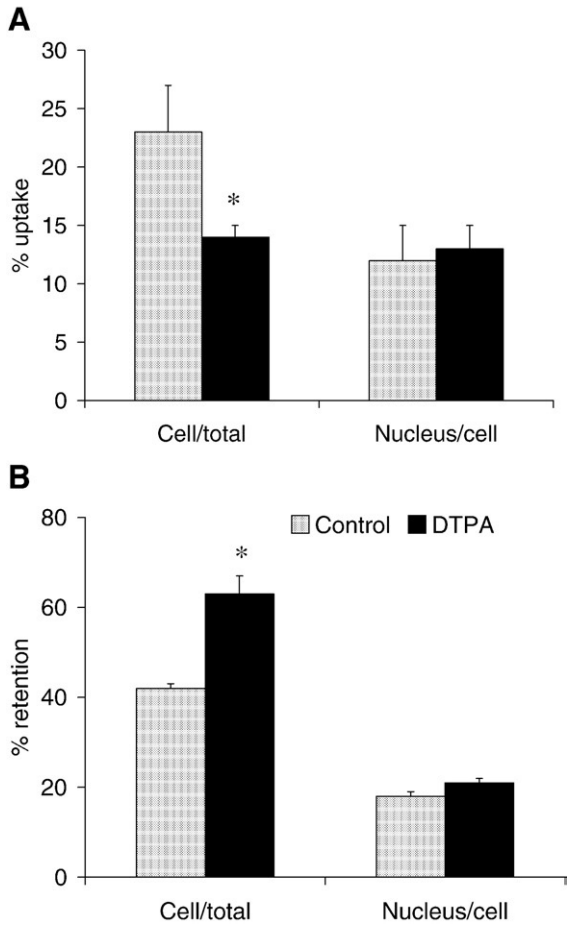

Fig. 3. Effect of DTPA on uptake (A) and retention (B) of ^{65}Zn in H4IIE cells. Cells were incubated with ^{65}Zn with or without DTPA (50 μM) for 48 h to measure zinc uptake. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. Values are the mean±S.D. of three experiments, each including at least four replicates of each treatment. The asterisk indicates significant difference from control, $P<001$.

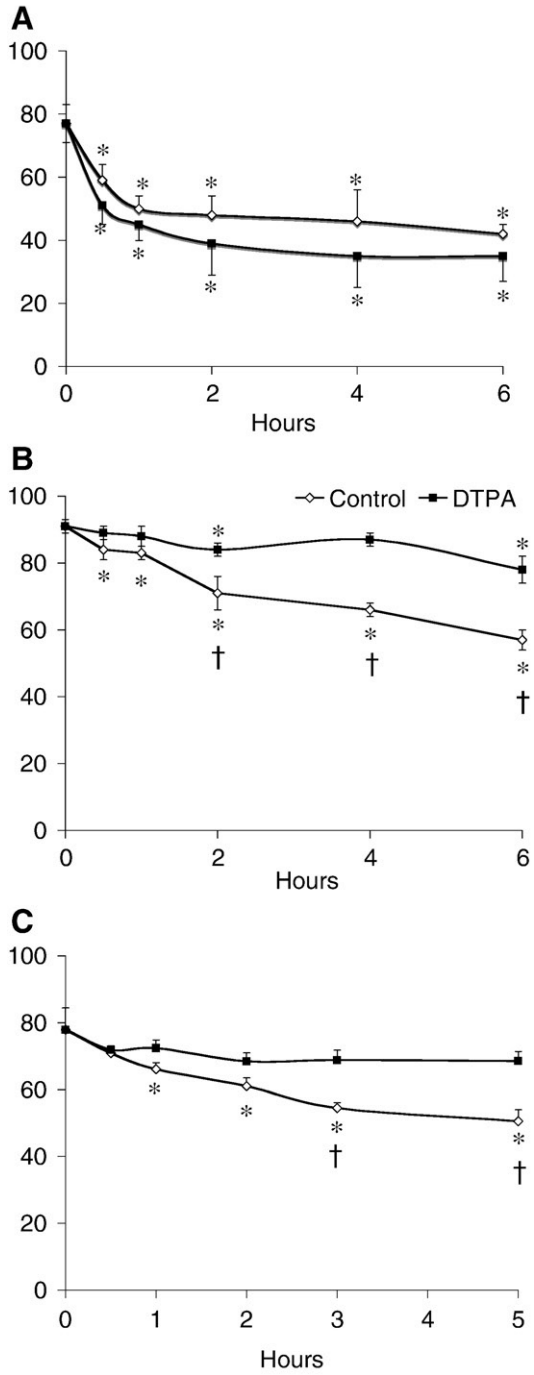

Fig. 4. Effect of DTPA on ^{65}Zn efflux over time in primary hepatocytes (A), H4IIE cells (B) and GH3 cells (C). Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for the times shown to measure retention. "% retention" represents the fraction of total recovered radioactivity at each time point found within the cells. Values are the mean±S.D. of four to six replicates, and each experiment was repeated at least once. The asterisk indicates significant difference from time 0, whereas the dagger indicates significant difference from control at the same time; $P<05$ for both.

significantly decreased by 1 h of incubation and continued to decrease gradually, reaching 40–60% of the starting amount after 5 or 6 h (Fig. 4). In primary rat hepatocytes (Fig. 4A), DTPA caused greater efflux of ^{65}Zn ($P<.001$ for treatment effect by ANOVA). At each time point, mean retention of ^{65}Zn appeared lower in DTPA-treated cells, although post hoc analysis did not reveal differences at any individual time. However, in the two tumor cell lines, DTPA reduced the efflux of zinc such that at most times it did not differ significantly from 0 time cells. In addition, significantly more ^{65}Zn was retained following DTPA treatment in H4IIE cells by 2 h (Fig. 4B) and in GH3 cells by 3 h (Fig. 4C) than in control cells. Thus, the effects of DTPA on zinc efflux can be observed in the first hours of treatment.

To further explore the differential effects of DTPA in transformed and nontransformed cells, we examined zinc flux in two human mammary cell lines, MCF-7 breast cancer cells and Hs578Bst normal epithelial breast cells. As with the other cells examined, DTPA reduced ^{65}Zn uptake by both MCF-7 (Fig. 5A) and Hs578Bst (Fig. 6A) cells. The extent of zinc uptake was lower, at about 5% as compared with

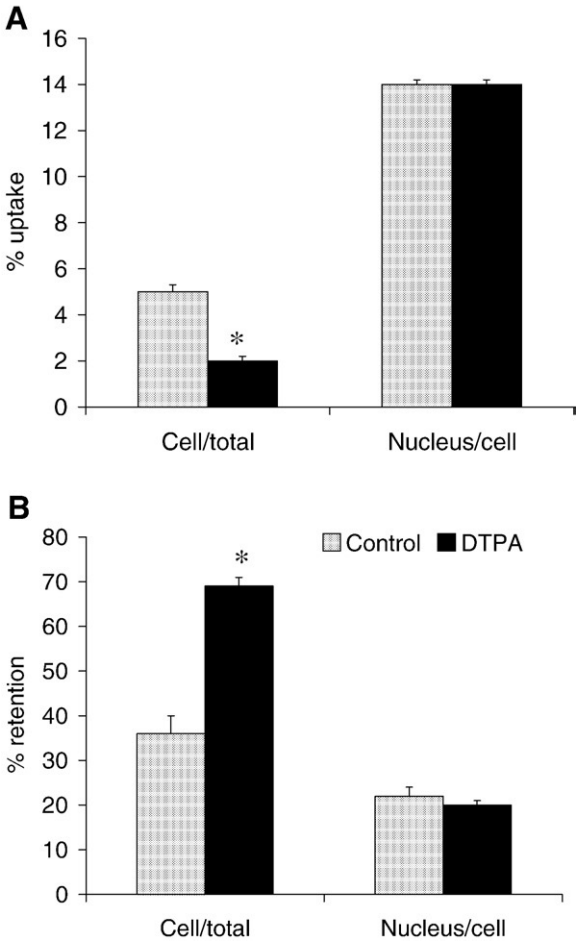

Fig. 5. Effect of DTPA on uptake (A) and retention (B) of ^{65}Zn in MCF-7 cells. Cells were incubated with ^{65}Zn with or without DTPA (50 μM) for 48 h to measure zinc uptake. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. Values are the mean±S.D. of five replicates, and similar results were seen in a second experiment. The asterisk indicates significant difference from control, $P<.001$.

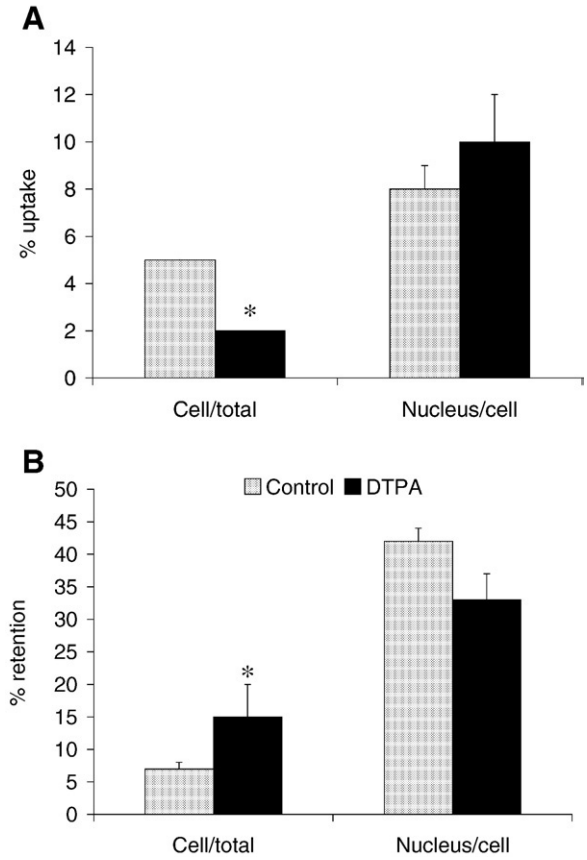

Fig. 6. Effect of DTPA on uptake (A) and retention (B) of ^{65}Zn in Hs578Bst cells. Cells were incubated with ^{65}Zn with or without DTPA (50 μM) for 48 h to measure zinc uptake. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. Values are the mean±S.D. of five replicates, and similar results were seen in a second experiment. The asterisk indicates significant difference from control, $P<.001$.

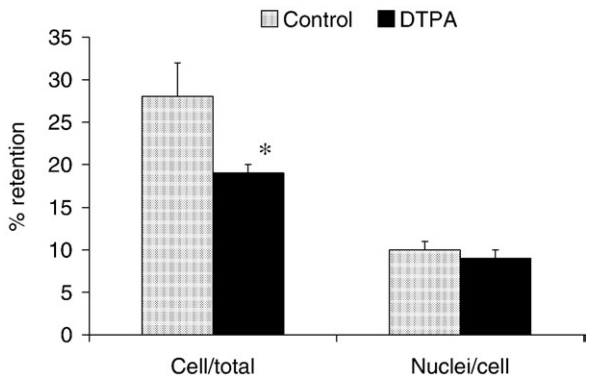

Fig. 7. Effect of DTPA on retention of ^{65}Zn in rat primary anterior pituitary cells. Cells were prelabeled with ^{65}Zn for 48 h, rinsed and incubated with or without DTPA for 48 h to measure retention. Values are the mean±S.D. of five replicates, and similar results were seen in a second experiment. The asterisk indicates significant difference from control, $P<.001$.

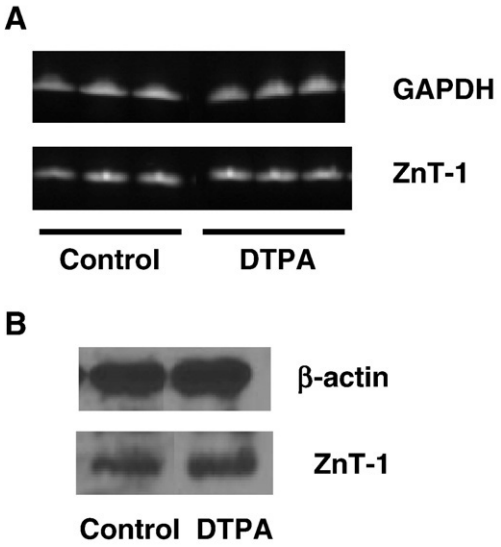

Fig. 8. Effect of DTPA on ZnT-1 mRNA (A) and protein (B) expressions in H4IIE cells. Cells were treated with or without DTPA (50 μM) for 48 h prior to extraction of RNA and protein. (A) ZnT-1 and GAPDH mRNA concentrations were measured by RT-PCR with ethidium bromide staining of products separated by agarose gel electrophoresis. (B) ZnT-1 and β-actin proteins were measured by Western blot analysis following SDS-PAGE separation of proteins. Representative lanes are shown, and no effect of treatment was found in three experiments with four replicates in each treatment group.

20–30% in other cell types studied. As expected, MCF-7 cells showed increased ^{65}Zn retention with DTPA, showing a substantial increase to 70% as compared with 30% in the control cells (Fig. 5B). Hs578Bst, which is not a cancer cell line, also showed an increased retention of ^{65}Zn with DTPA to about 15% as compared with 7% in the control cells (Fig. 6B). This also indicated that the retention of zinc in Hs578Bst was lower compared with other cell types.

Hs578Bst cells were isolated from normal breast tissue located peripheral to an infiltrating ductal carcinoma [15] and represent an actively growing cell line rather than true primary cells. Therefore, the effect of DTPA on zinc efflux was also examined in primary anterior pituitary cells (Fig. 7). In this case, cells incubated with DTPA retained 15% of the ^{65}Zn, as compared with 28% in the control cells. DTPA did not affect the fraction of cellular radioactive zinc found in the crude nuclear pellet.

Both mRNA and protein concentrations of ZnT-1, the zinc efflux transporter, were measured in H4IIE cells that had been treated with or without DTPA for 48 h. DTPA had no effect on ZnT-1 mRNA or protein concentrations when corrected for expression of appropriate housekeeping genes (Fig. 8).

4. Discussion

DTPA has been used by us [12,13,16] and others [5] to reduce zinc availability to cultured cells based on the assumption that it does not cross cell membranes. This is in contrast to such chelators as TPEN (*N*,*N*,*N*′,*N*-tetrakis(-)[2-pyridylmethyl]-ethylenediamine) that do enter the cell and cause more profound disruptions in zinc homeostasis. However, data demonstrating the membrane impermeability of DTPA could not be found in the literature. Consequently, ^{14}C-labeled DTPA was obtained to determine whether it could be taken up by cultured cells. After 48 h of incubation, virtually all of the labeled DTPA was recovered from the medium, confirming that this chelator is membrane impermeable and that its actions are therefore limited to the outside of the cell.

The ability of DTPA to bind ^{65}Zn in the medium and reduce its availability for cellular uptake was confirmed in all cells examined. The extent of the reduction in uptake varied across cell types; for example, DTPA reduced uptake by GH3 cells to a much greater extent than it did with H4IIE cells. However, in all cases, less ^{65}Zn entered the cells in the presence of DTPA than in its absence.

When efflux of ^{65}Zn was measured, cultured cells varied in their response to zinc deprivation. At least in the liver and pituitary, this differential response appeared to be associated with the primary versus transformed nature of the cells. Under the influence of DTPA, all the transformed cells in the study, including GH3, H4IIE and MCF-7, exhibited an increased retention of zinc as shown by reduced ^{65}Zn efflux. These results were surprising because DTPA, being an extracellular metal chelator, was expected to pull zinc out of the cells by binding to the zinc in the medium and creating a concentration gradient favoring zinc efflux. On the other hand, both primary hepatocytes and primary pituitary cells gave up more ^{65}Zn to the medium in the presence of DTPA, thus exhibiting the predicted response to zinc chelation. However, Hs578Bst, a cell line established from normal mammary tissue, also showed an increased retention of radioactive ^{65}Zn with DTPA, a response similar to that of transformed cells and different from that of primary cells.

The enhanced retention of zinc in response to reduced zinc availability seen with the cell lines could be associated with its importance for maintaining cell division. Zinc is actively involved in the various stages of cell proliferation. Cells cannot proliferate in zinc-free medium, and growth is arrested in the S-phase of the cell cycle [17]. Zinc supplementation promotes DNA synthesis, whereas zinc depletion suppresses it [18]. Therefore, it is possible that transformed cells have developed a mechanism to restrict zinc efflux under conditions of zinc deficiency in order to preserve intracellular concentrations and continue their proliferation. The similarity in the response of Hs578Bst cells to the cancer lines could be explained by the fact that, while it is not transformed, it is an established cell line that continues to replicate. Thus, with respect to growth regulation, they may be more similar to the cancer cell lines than primary cells. This would suggest an enhanced requirement for zinc and therefore the homeostatic response of reduced zinc efflux in response to DTPA observed here.

While zinc restriction is likely to impair cellular growth, changes in cell number are unlikely to explain the alterations in zinc flux seen here. Cell lines were used under near-confluent conditions, and only minor alterations in cell number or viability were detected. Furthermore, since total cellular retention of zinc was enhanced by DTPA, any reduction in cell number would actually exaggerate rather than explain this effect. In addition, the time course studies showed effects of DTPA in the first hours of treatment, well before any proliferative effect is likely to be manifest.

The DTPA is in the medium, and therefore its prime effect is to bind zinc there and reduce cellular uptake. In the cell lines, there is a subsequent drop in zinc efflux, presumably part of a homeostatic attempt to maintain cellular zinc. At this time, it is unclear whether this is successful (i.e., whether the reduction in efflux can make up for the drop in influx). However, the fact that this reduction in efflux is seen suggests that, in some way, the cells are sensing zinc deprivation and responding to it. These experiments clearly demonstrate that either the mechanism for perceiving zinc status differs between transformed and primary cells or different signals are elicited in these cell types after zinc deficiency is detected.

Zinc status is governed in cells by a number of zinc transporters, metallothionein and zinc-sensing proteins, although understanding of the mechanisms of action and regulation of the transporters is limited [19]. The only plasma membrane zinc efflux transporter that has been identified up until now is ZnT-1. Measurements of mRNA expression and protein expression demonstrated that DTPA had no effect on the amount of ZnT-1 in H4IIE cells. This is consistent with the ^{65}Zn time course experiments, which showed that DTPA acts rapidly to reduce zinc efflux in both GH3 and H4IIE cells. Thus, while not influencing the amount of ZnT-1 protein in cells, DTPA could alter its cellular location or its activity in a cell-specific manner. Alternatively, other less characterized transporters may be responsible for the differential efflux responses seen here.

Except for the primary hepatocytes, DTPA did not affect the proportion of ^{65}Zn found in the crude nuclear fraction. Thus, as cellular zinc content varied, nuclear zinc changed in the same way such that it comprised a constant proportion of total cellular zinc. However, in primary hepatocytes, DTPA disproportionately increased efflux of zinc from the crude nuclear fraction such that it formed a reduced proportion of cellular zinc. While the procedure for isolating nuclei may allow the inclusion of other heavy cell debris, this does indicate the possibility of independent regulation of nuclear zinc homeostasis in primary hepatocytes. In all other cells examined, nuclear ^{65}Zn simply followed the cellular concentrations.

The differences in growth and proliferation between transformed cell lines and normal tissues as well as the primary cells derived from them are clearly established. However, transformed cell lines have been used extensively as good models for understanding the mechanisms of a wide range of cellular processes. This current work demonstrates the need for caution in assuming that tumor cell lines mimic normal metabolism because, at least with respect to zinc homeostasis, they do not. However, it also suggests that alterations in the processes governing the transport of zinc may be important for these rapidly growing cells and that understanding the underlying mechanisms may give greater insight into their deregulated growth.

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