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Zinc transport by respiratory epithelial cells and interaction with iron homeostasis

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Abstract Despite recurrent exposure to zinc through inhalation of ambient air pollution particles, relatively little information is known about the homeostasis of this metal in respiratory epithelial cells. We describe zinc uptake and release by respiratory epithelial cells and test the postulate that Zn^{2+} transport interacts with iron homeostasis in these same cells. Zn^{2+} uptake after 4 and 8 h of exposure to zinc sulfate was concentration- and time-dependent. A majority of Zn^{2+} release occurred in the 4 h immediately following cell exposure to $ZnSO_4$. Regarding metal importers, mRNA for Zip1 and Zip2 showed no change after respiratory epithelial cell exposure to zinc while mRNA for divalent metal transporter (DMT)1 increased. Western blot assay for DMT1 protein supported an elevated expression of this transport protein following zinc exposure. RT-PCR confirmed mRNA for the metal exporters ZnT1 and ZnT4 with the former increasing after $ZnSO_4$. Cell concentrations of ferritin increased with zinc exposure while oxidative stress, measured as lipid peroxides, was decreased supporting an anti-oxidant function for Zn^{2+}. Increased DMT1 expression, following pre-incubations of respiratory epithelial cells with TNF-α, IFN-γ, and endotoxin, was associated with significantly decreased intracellular zinc transport. Finally, incubations of respiratory epithelial cells with both zinc sulfate and ferric ammonium citrate resulted in elevated intracellular concentrations of both metals. We conclude that exposure to zinc increases iron uptake by respiratory epithelial cells. Elevations in cell iron can possibly affect an increased expression of DMT1 and ferritin which function to diminish oxidative stress. Comparable to other metal exposures, changes in iron homeostasis may contribute to the biological effects of zinc in specific cells and tissues.

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Abbreviations

DMSO	Dimethylsulfoxide
DMT1	Divalent metal transporter 1
DNPH	2,4-Dinitrophenylhydrazine
DPBS	Dulbecco's phosphate buffered saline
HBE	Human bronchial epithelial
ICPOES	Inductively coupled plasma optical emission spectroscopy
KGM	Keratinocyte growth medium
LDH	Lactate dehydrogenase
MTT	Methylthiazoletetrazolium
PM	Particulate matter
TCA	Trichloroacetic acid
Tf	Transferrin
TfR	Transferrin receptor

Introduction

Zinc is present in all organs, tissues, and body fluids. In multicellular organisms, virtually all of this metal is intracellular (Tapiero and Tew 2003; Vallee and Falchuk 1993). It is an essential micronutrient involved in numerous structural and regulatory cell functions. In the cell, Zn^{2+} functions in metalloenzymes and transcription factors and is an important immunoregulatory agent, growth cofactor, and cytoprotectant with anti-oxidant, anti-apoptotic, and anti-inflammatory roles (Chai et al. 1999; Zalewski et al. 2005). As a result of the absolute requirement for zinc, all living systems evolved complex and frequently redundant mechanisms for its regulation (Colvin et al. 2003). Zinc homeostasis is influenced by numerous endogenous hormones, cytokines, and growth factors (Costello et al. 1999; Rooprai et al. 2000). Perturbations of this homeostasis can include increased uptake, excessive losses, and redistribution of the metal in a cell, tissue, and living system.

In addition to zinc taken up from food by the gastrointestinal tract to meet metabolic requirements, the human respiratory tract is exposed to this metal which is frequently included in air pollution particles. Specific sources of atmospheric zinc include industrial processes and tire wear (Boughton and Horvath 2004; Councell et al. 2004; Falahi-Ardakani 1984; Yaghi and Abdul-Wahab 2003). Epidemiological investigation has associated inhalation of particulate matter (PM) with adverse health effects including pulmonary and cardiovascular disease and mortality (Dockery et al. 1993; Koenig et al. 1993; Schwartz 1994; Samet et al. 2000). Cell, animal, and human investigation has demonstrated a potential for zinc included in PM to contribute to adverse pulmonary and systemic health effects, including pulmonary inflammation and hematological changes, after exposure to ambient air pollution particles (Gordon et al. 1992; Clarke et al. 2000; Prieditis and Adamson 2002; Riley et al. 2003).

Properties of zinc are of particular importance in the epithelial tissues which are at the front line of lung defense (Zalewski et al. 2005). However, relatively little information is known about the import and export of zinc by human bronchial epithelial cells. We describe zinc uptake and release by both BEAS-2B cells, a human bronchial epithelial cell line, and primary human bronchial epithelial cells.

Materials and methods

Materials

Tissue culture media, supplements, and supplies were from Clonetics (San Diego, CA). Dulbecco's phosphate buffered saline (DPBS) was purchased from GibcoBRL (Gaithersburg, MD). TaqMan Universal PCR Master Mix was obtained from Roche (Branchburg, NJ), RNeasy Kit from QIAGEN Inc. (Valencia, CA) and RiboGreen RNA Quantitation Kit from Molecular Probes. Maloney murine leukemia virus reverse transcriptase, NuPAGE gels, reducing agent, antioxidant, and loading, running and transfer buffers were from Invitrogen (Calsbad, CA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphatase inhibitor cocktail sets I and II and protease inhibitor cocktail set III were purchased from Calbiochem (San Jolla, CA) and ECL from Amersham Pharmatia Biotech (Piscataway, New Jersey). Human tumor necrosis factor (TNF)-α and interferon (IFN)-δ were from R & D Systems (Minneapolis, MN). Endotoxin was *E. coli* O55:B5 from Sigma Co. (St. Louis, MO). Bovine serum

albumin (BSA) and all other laboratory chemicals were purchased from Sigma Chemical (St. Louis, MO) unless specified otherwise.

Culture of human respiratory epithelial cells

BEAS-2B cells were used in all studies. This is an immortalized line of normal human bronchial epithelium derived by transfection of primary cells with SV40 early region genes. Cells were grown to 90–100% confluence on uncoated plastic twelve-well plates in keratinocyte growth medium (KGM) which is essentially MCDB 153 medium supplemented with 5 ng/ml human epidermal growth factor, 5 mg/ml insulin, 0.5 mg/ml hydrocortisone, 0.15 mM calcium, bovine pituitary extract, 0.1 mM ethanolamine, and 0.1 mM phosphoethanolamine.

To confirm zinc and iron uptake and release, human bronchial epithelial cells (HBE) were obtained from healthy, non-smoking adult volunteers by cytologic brushing of the airways during bronchoscopy. These cells were expanded to passage-3 in bronchial epithelial growth medium (BEGM) and plated at a density of 1×105 cells/well into 12 well culture plates. Cells were grown to 90–100% confluence on plastic twelve-well plates in bronchial epithelial growth medium (BEGM) which is LHC-9 medium. Fresh medium was provided every 48 h.

Regarding the media used in this investigation, KGM has both zinc and iron at concentrations of 0.1 ± 0.1 and 4.1 ± 0.3 μM while levels in BEGM are 3.0 ± 0.1 and 2.8 ± 0.1 μM, respectively.

Cytotoxicity assay

Lactate dehydrogenase (LDH) release and methylthiazoletetrazolium (MTT) reduction were employed as measures of cytotoxicity. Respiratory epithelial cells were grown to 90–100% confluence on 12-well plates and exposed to $ZnSO_4$ in KGM for 24 h. LDH concentration in the cell supernatant was measured using a commercially prepared kit (Sigma) as modified for automated measurement (Cobas Fara II centrifugal analyzer). In addition, MTT (500 μg/ml) was added to each well. The plate was returned to the incubator (37°C) for 1–3 h. After removal of the supernatant, the cells were washed with DPBS and 500 μl dimethylsulfoxide (DMSO) was added to each well to dissolve the formazine. Absorbance of the formazine was measured at a wavelength of 570 nm.

Cellular uptake of zinc

The effect of the initial $ZnSO_4$ concentration on metal uptake by the respiratory epithelial cells was studied. Cells were grown in 12-well plates to 90–100% confluence in either KGM or BEGM. Medium was switched to DPBS and cells were exposed to 0, 5, 10, and 25 μM $ZnSO_4$ for 4 and 8 h. Supernatant was removed and the cells were washed twice with DPBS. Cell lytates were scraped into 125 μl RIPA buffer and 1.0 ml of 3N HCl with 10% trichloroacetic acid (TCA) added. Cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at $20{,}800 \times g$ for 5 min, zinc concentration in the supernatant was determined using inductively coupled plasma optical emission spectroscopy (ICPOES).

The effect of incubation time on metal uptake was evaluated. Cells were grown in 12-well plates to 90–100% confluence in either KGM or BEGM. Medium was switched to DPBS and cells were exposed to 25 μM $ZnSO_4$ for 15, 30, 45 min, 1, 4, and 8 h. After lysis with 125 μl RIPA buffer, the cells were hydrolyzed in 1.0 ml of 3N HCl with 10% TCA at 70°C for 18 h. Following centrifugation at $20{,}800 \times g$ for 5 min, zinc concentration in the supernatant was determined using ICPOES.

To discriminate between intracellular and surface bound concentrations of zinc, cells were exposed to the metal and either directly scraped into acid or incubated with trypsin/inhibitor, centrifuged, washed, and then treated with acid. BEAS-2B cells were grown in a 12-well plate to 90–100% confluence in KGM. Medium was switched to DPBS and cells were exposed to 0 and 25 μM $ZnSO_4$ for 8 h. Supernatant was removed and cells were washed twice with DPBS. Six wells of each plate (3 exposed to DPBS and 3 exposed to 25 μM $ZnSO_4$) were scraped into 1.0 ml 3N HCl/10% trichloroacetic acid and the cells hydrolyzed at 70°C for 24 h and zinc concentration determined using ICPOES. Another six wells of the same plate were similarly exposed but then incubated with 0.25% trypsin/EDTA (Gibco, Carlsbad, CA) until the cells were observed to be dislodged from the plate. An equal volume of soybean trypsin inhibitor (Sigma) was then added and the suspended cells were centrifuged. After removal of the trypsin/EDTA and

inhibitor, the cells were washed and 1.0 ml 3N HCl/10% trichloroacetic acid added. Hydrolysis at 70°C for 24 h followed and zinc concentration was determined using ICPOES. The experiment was repeated twice."

Cellular release of zinc

Zinc release by BEAS-2B and human bronchial epithelial cells was evaluated. Cells were grown to 90–100% confluence in either KGM or BEGM, medium was switched to DPBS, and cells were exposed to 25 μM $ZnSO_4$ for 8 h. DPBS was removed, the cells were washed, and 1.0 ml complete media was added to each well of cells. After incubation for 30 min, 1, 2, 4, 8, or 24 h in the media, supernatant was removed, cells were washed twice, scraped into 125 μl RIPA buffer, and 1.0 ml of 3N HCl with 10% TCA was added. Cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20,800×*g* for 5 min, zinc in the supernatant was measured using ICPOES.

Measurement of zinc and iron concentrations

Zinc and iron concentrations were quantified using ICPOES (Model Optima 4300D, Perkin Elmer, Norwalk, CT) at wavelengths of 206.200 and 238.204 nm, respectively. A multi-element standard (Spex Certiprep, Metuchen, NJ) was used to calibrate the instrument. The limits of detection approximated 1 and <0.1 ppb, respectively.

RT-PCR for proteins involved in zinc transport

BEAS-2B cells were grown to 90% confluence and exposed to 0, 5, 10, and 25 μM $ZnSO_4$ for 8 h. Cells were lysed with 4 M guanidine thiocyanate, 50 mM sodium citrate, 0.5% sarkosyl, and 0.01 M dithiothreitol. After the cells were dislodged from wells with scrapers, lysates were sheared with four passes through a 22-gage needle. RNA was isolated using RNeasy Kit and RNA concentration was measured using RiboGreen RNA Quantitation Kit. Total RNA (200 ng) was reversely transcribed into cDNA. Quantitative PCR was performed on a sequence detector (Applied Biosystems 7500 Real Time PCR Systems, Applied Biosystems, Foster City, CA). ZIP1, ZIP2, DMT1, ZnT1, ZnT2, and ZnT4 were quantified using SYBR Green Universal PCR Master Mix (Applied Biosystems) and oligonucleotide primer pairs designed using a primer design program (Primer Express, Applied Biosystems). Finally, mRNA levels were measured using Taqman Universal PCR Master Mix (Applied Biosystems) with primer/probe sets obtained as pre-optimized mixes ("Assays-on-Demand" from Applied Biosystems). mRNA levels were normalized to expression of β-actin as a reference gene.

Western blot assay for DMT1 expression

BEAS-2B cells were exposed to either KGM or $ZnSO_4$ for 24 h, washed, lysed with RIPA buffer containing 1% NP40, 0.5% deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail sets I and II and protease inhibitor cocktail Set III (Calbiochem, La Jolla, CA), and sheared through a 22-g needle. Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA). The remainder of the sample was mixed with an equal volume of 4× sample loading buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 mM β-mercaptoethanol, 0.05% bromphenol blue). Protein samples (50 mg) were separated by electrophoresis on a 4–15% SDS acrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 3% non-fat milk in DPBS and incubated with an antibody directed against divalent metal transporter 1 (DMT1). The membrane was stained with a horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and developed using enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech).

Ferritin, transferrin, and transferrin receptor after zinc exposure

Cell lysates were collected after 24 h exposure to either KGM or varying concentrations of $ZnSO_4$ and analyzed for ferritin, transferrin, and transferrin receptor. Supernatant was removed and the cells washed with DPBS. Cells were scraped into 0.5 ml DPBS and this lysate sheared with four passes through a 22 gage needle. Ferritin and transferrin receptor (TfR) concentrations were measured with commercially available ELISA kits (R & D Systems,

Minneapolis, MN). Transferrin protein concentrations in the lysate were analyzed using a commercially available kit, controls, and standards from INCSTAR Corporation (Stillwater, MN). These assays were adapted for use on the Cobas Fara II centrifugal spectrophotometer.

Oxidative stress in BEAS-2B cells following exposure to zinc

Following 8 h of exposure to 0, 5, 10, and 25 μM $ZnSO_4$, cells were washed with DPBS, scrapped into 1.0 ml 2,4-dinitrophenylhydrazine (DNPH) solution (0.125% in acetonitrile), and vortexed. Normal saline added to DNPH was used as a blank. Acetaldehyde concentrations were quantified as an index of oxidative stress using high performance liquid chromatography (Madden et al. 1999).

Zinc uptake following endotoxin and cytokine exposures

Human respiratory epithelial cells increase expression of DMT1 following exposure to endotoxin, TNF-α, and IFN-γ, and endotoxin (Wang et al. 2005). Iron uptake is similarly increased in these cells following such exposures (Wang et al. 2005). BEAS-2B cells were exposed for 4 h to 100 μg/ml endotoxin, 50 ng/ml TNF-α, and 100 ng/ml IFN-γ. mRNA for DMT1 was determined using RT-PCR. Cell exposures to endotoxin, TNF-α, and IFN-γ were repeated but incubations continued for 24 h. Medium was then switched to DPBS and cells were exposed to 0 and 25 μM $ZnSO_4$ for 8 h. Supernatant was removed and the cells were washed twice with DPBS. Cell lytates were scraped into 125 μl RIPA buffer and 1.0 ml of 3 N HCl with 10% TCA. Cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20,800×*g* for 5 min, zinc concentration in the supernatant was determined using ICPOES.

Zinc and iron uptake with co-exposures

The effect of co-exposures on zinc and iron uptake by the respiratory epithelial cells was studied. Cells were grown in 12-well plates to 90–100% confluence in KGM. Medium was switched to DPBS and cells were exposed to 100 μM FAC and 25 μM zinc sulfate for 8 h. Supernatant was removed and the cells were washed twice with DPBS. Cell lytates were collected in 125 μl RIPA buffer and 1.0 ml of 3N HCl with 10% TCA. These cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20,800×*g* for 5 min, zinc concentrations in the supernatant were determined using ICPOES.

Cells were exposed to 25 μM $ZnSO_4$ and 100 μM FAC in DPBS for 4 h. Supernatant was removed, the cells were washed twice with DPBS, and cell lytates were collected in 125 μl RIPA buffer and 1.0 ml of 3N HCl with 10% TCA. These cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20,800×*g* for 5 min, iron concentrations in the supernatant were determined using ICPOES.

Statistics

Data are expressed as mean value ± standard deviation. Differences between multiple groups were compared using one-way ANOVA test. The post-hoc test employed was Scheffe's test. Two-tailed tests of significance were employed. Significance was assumed at $P < 0.05$.

Results

BEAS-2B and human bronchial epithelial cells were exposed to varying concentrations of $ZnSO_4$ in media for 24 h. Using LDH release and MTT reduction, there was no evidence of cytotoxicity following exposures of BEAS-2B and human bronchial epithelial cells to zinc sulfate at concentrations ≤25 μM.

Intracellular transport of Zn^{2+} by BEAS-2B cells after 4 and 8 h exposure to zinc sulfate was concentration-dependent (Fig. 1a). Import of this metal by these same cells was also time-dependent (Fig. 1b). Zinc uptake by human respiratory epithelial cells was similarly both concentration- (Fig. 1c) and time-dependent (Fig. 1d).

At 8 h, cells exposed to DPBS and 25 μM $ZnSO_4$ and directly scraped into acid revealed zinc concentrations of 0.138 and 1.465 μM, respectively. Treatment with trypsin/inhibitor of cells following the same exposures showed zinc levels of 0.121 and 1.258 μM. The small differences may reflect less than total displacement of all cells from the plate with trypsinization or cell loss during centrifugation.

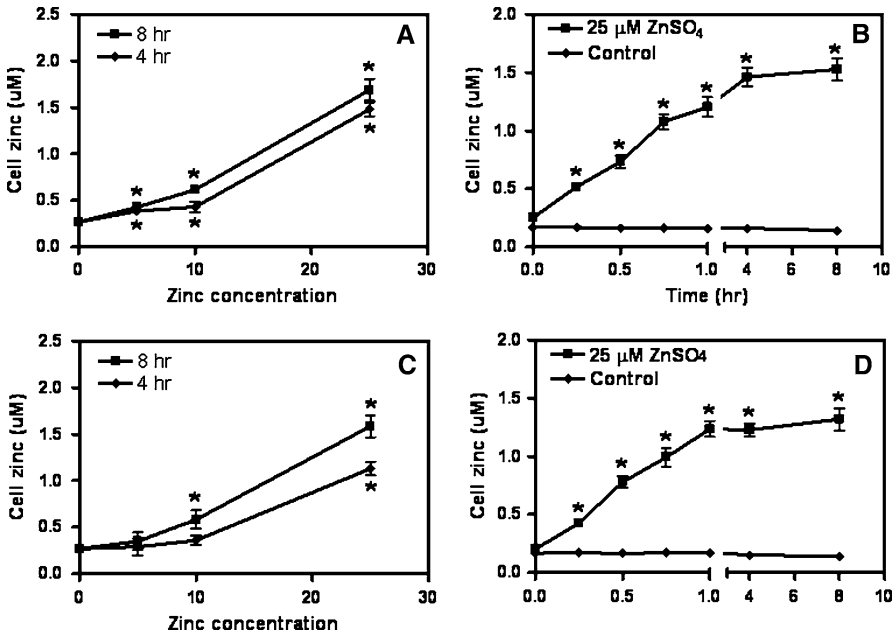

Fig. 1 Exposures to $ZnSO_4$ increase cell zinc uptake in a concentration- and time-dependent manner. BEAS-2B cells were grown in 12-well plates to 90–100% confluence in KGM. Medium was switched to DPBS, and cells were exposed to 0, 5, 10, and 25 μM Zn^{2+} for 4 and 8 h (**a**) and to 25 μM Zn^{2+} for up to 8 h (**b**). Human bronchial epithelial cells, initially in BEGM but switched to DPBS, were similarly exposed (**c** and **d**). After exposures, supernates were removed, and cells were washed twice with DPBS. Lytates were collected in 125 μl RIPA buffer and 1.0 ml of 3N HCl with 10% TCA. Cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20,800×*g* for 5 min, zinc concentration was determined using ICPOES ($\lambda = 206$ nm). The results provide the mean ± standard deviation of a representative of three or more experiments. $^*P < 0.05$ compared with exposure to either buffer only or time = 0 h

These results support the zinc being truly intracellular rather than any metal adherence to the cell surface.

Unlimited cell uptake of zinc is unlikely to benefit the respiratory epithelial cells and some mechanism of metal export is predicted. Exposure of respiratory epithelial cells to $ZnSO_4$ 25 μM for 8 h with subsequent removal to media resulted in metal release (Fig. 2). The majority of metal release occurred in the hours immediately following loading of the cells with zinc. However, even after 24 h in medium, cell zinc concentrations were significantly elevated above baseline indicating that not all of the imported Zn^{2+} was released.

BEAS-2B cells were analyzed for expression of common zinc transporters as a result of a demonstrated capacity for zinc uptake and release. mRNA for Zip1 and Zip2 in these respiratory cells were quantified 8 h after exposure to varying concentrations of zinc sulfate. Quantitative RT-PCR confirmed the presence of both transporters in respiratory epithelial cells prior to zinc exposure (Fig. 3a). There was approximately ten-fold more mRNA for Zip1 than Zip2. Following $ZnSO_4$ exposure, there were no changes in the mRNA levels demonstrated for either protein suggesting a lack of involvement in the import of this metal. Alternatively, Zip1 and Zip2 could be involved in zinc uptake by these cells but post-transcriptional mechanisms for control of protein expression could be involved. Comparable to other investigation (Wang et al. 2002), DMT1 mRNA was demonstrated by quantitative RT-PCR to be present in these respiratory epithelial cells and it increased with zinc exposure (Fig. 3b). Quantification of DMT1 protein by Western blot assay supported increased protein expression following zinc exposure (Fig. 3c). Using RT-PCR, expression of the zinc exporters ZnT1, ZnT2, and ZnT4 was analyzed. No mRNA for ZnT2 was found and BEAS-2B cells do not appear to express this specific transporter. RT-PCR confirmed mRNA for both ZnT1 and ZnT4 (Fig. 4). ZnT1 showed a dose-dependent increase in mRNA suggesting its possible

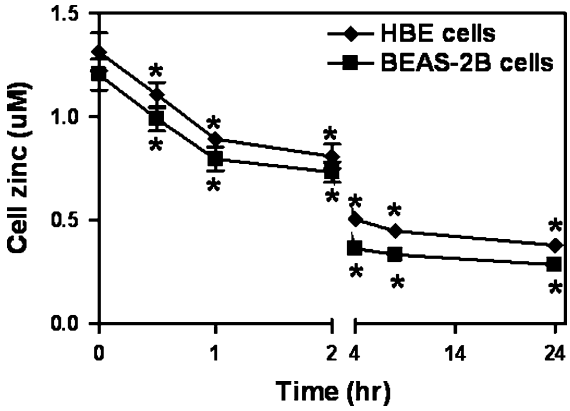

Fig. 2 Zinc is released following treatment with 25 μM Zn^{2+} in DPBS. BEAS-2B and human bronchial epithelial cells were grown to 90–100% confluence in KGM and BEGM, respectively, medium was removed, DPBS added, and cells were exposed to 25 μM Zn^{2+} for 8 h. The buffer (with and without Zn^{2+}) was removed, and cells were washed with DPBS. One milliliter medium (KGM and BEGM to BEAS-2B and human bronchial epithelial cells, respectively) was added to each well. After incubation for 30 min, 1, 2, 4, 8, or 24 h, supernates were removed. Cells were scraped into 125 μl of RIPA buffer, and 1.0 ml of 3N HCl with 10% TCA. Cell extracts were hydrolyzed at 70°C for 18 h. After centrifugation at 20800 × *g* for 5 min, zinc was measured using ICPOES. $^*P < 0.05$ compared with exposure to time = 0 h

involvement in Zn^{2+} export by respiratory epithelial cells.

Iron transport by DMT1 in respiratory epithelial cells is coupled with its storage in ferritin which provides some control of the oxidative stress presented by this metal (Turi et al. 2004). An increase in DMT1 following zinc exposure suggested a potential upregulation of a pathway of metal transport by respiratory epithelial cells involving control of oxidative stress (Wang et al. 2002, 2005). Therefore, concentrations of ferritin, transferrin, and transferrin receptor in BEAS-2B cells were measured after 24 h of BEAS-2B cells exposure to varying concentrations of zinc. Cell concentrations of ferritin increased with zinc exposure (Fig. 5). Concentrations of transferrin showed no changes with exposure to zinc (5.2 ± 0.8 μg/dl) while levels of transferrin receptor were below the limit of detection. To further assess the consequence of this metal transport pathway in zinc homeostasis, lipid peroxides were quantified to provide a measure of oxidative stress. Previous investigation has demonstrated that interventions increasing metal transport via DMT1 can result in an anti-oxidant effect (Ghio et al. 2005). Comparable to several other studies which have

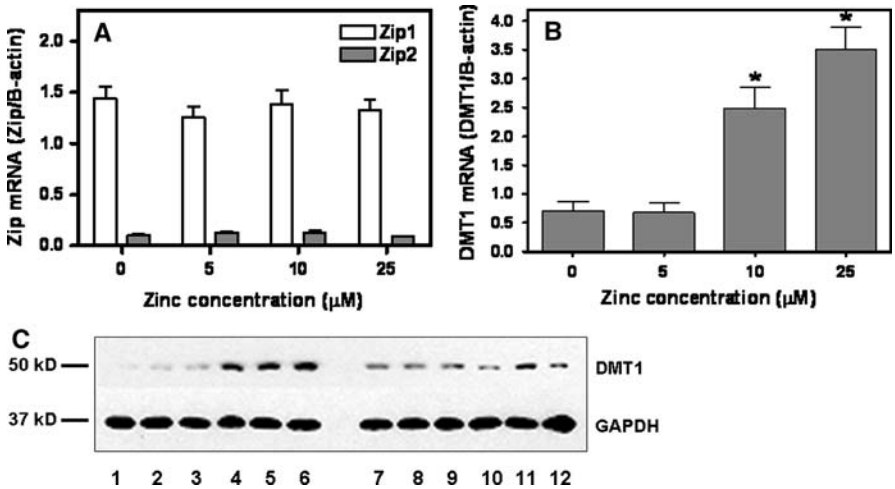

Fig. 3 Zn^{2+} increases mRNA for DMT1 and its protein expression in respiratory epithelial cells. BEAS-2B cells were grown in KGM and exposed to 0, 5, 10, and 25 μM Zn^{2+} for 6 h. Total mRNA was extracted using RNease kit and reserve-transcribed into cDNA. Quantitative RT-PCR was performed using TaqMan polymerase with green fluorescence on a sequence detector. Zip1, Zip2, and DMT1 mRNA levels were normalized by using the expression of *β*-actin as a reference gene and shown as fold change over control (**a** and **b**). Western blot assay for DMT1 demonstrated a comparable elevated protein expression with exposure of the BEAS-2B cells to increasing concentrations of zinc (**c**). Lanes 1–3, 4–6, 7–9, and 10–12 are cell incubations with 0, 25, 10, and 5 μM $ZnSO_4$, respectively. The results in are mean ± standard deviation (**a** and **b**). $^*P < 0.05$ compared with exposure to buffer only (**a** and **b**)

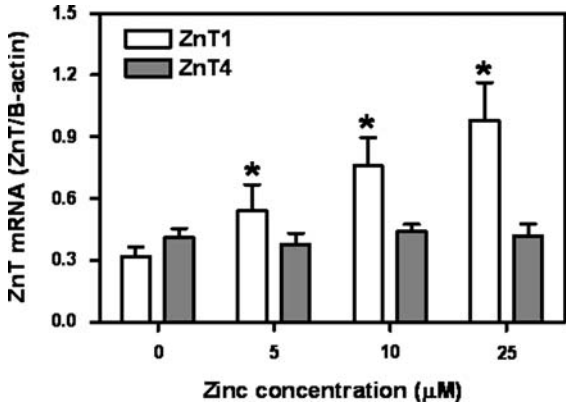

Fig. 4 Zinc induces dose-dependent ZnT1 expression in BEAS-2B cells treated with Zn^{2+}. BEAS-2B cells were grown in KGM and exposed to 0, 5, 10, and 25 μM Zn^{2+} for 6 h. mRNA extraction and quantitative RT-PCR for ZnT1 and ZnT2 were performed as described for Zip1, Zip2, and DMT1. Results are mean ± standard deviation. $^*P < 0.05$ compared with exposure to buffer only

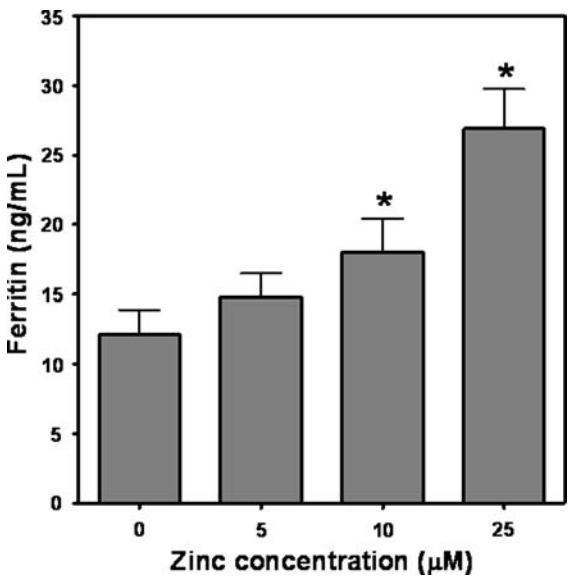

Fig. 5 Ferritin in BEAS-2B cells is increased after 24 h of exposure to Zn^{2+}. Cells were exposed to 0, 5, 10, and 25 μM $ZnSO_4$. Twenty-four hours later, they were washed with DPBS, scraped, and sheared with four passes through a 22 gage needle. Ferritin was measured with a commercially available ELISA kit. Results are mean ± standard deviation. $^*P < 0.05$ compared with exposure to buffer only

characterized zinc to be an anti-oxidant (Powell 2000), respiratory epithelial cells exposed to $ZnSO_4$ showed decreased concentrations of lipid peroxides (Fig. 6).

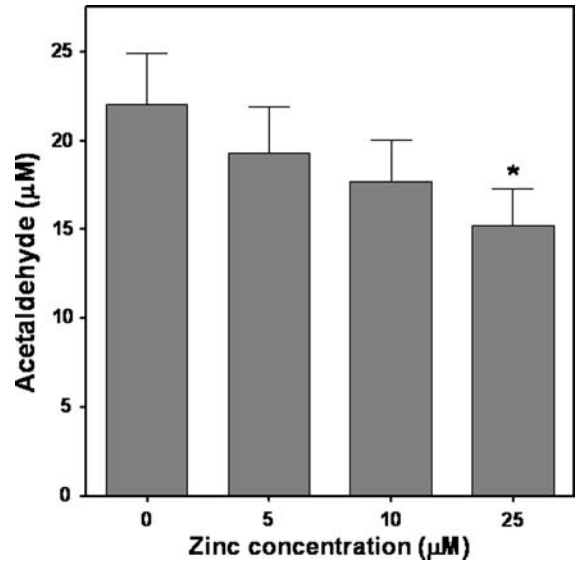

Fig. 6 Zinc decreases lipid peroxidation. BEAS-2B cells were exposed to 0, 5, 10, 25 μM Zn^{2+} in DPBS for 8 h. Supernates was removed and 1.0 ml added to 1.0 ml 2,4-dinitrophenyhydrazine (DNPH) solution (0.125% in acetonitrile). Cells were washed twice with DPBS, scrapped into DNPH solution, and vortexed. DNPH was extracted and analyzed by HPLC-MS. $^*P < 0.05$ compared with exposure to buffer only

TNF-α, IFN-γ, and endotoxin increase both the expression of DMT-1 in respiratory epithelial cells and cell uptake of iron (Wang et al. 2005). Four hour incubations of BEAS-2B cells with TNF-α, IFN-γ, and endotoxin significantly increased mRNA for DMT1 with DMT1/β actin values of 1.8 ± 0.4, 3.9 ± 0.7, and 3.4 ± 0.8, respectively (media only exposure showed a DMT1/β actin value of 0.9 ± 0.3). However, rather than increasing zinc uptake by the cell, pre-incubations of the respiratory epithelial cells with TNF-α, IFN-γ, and endotoxin significantly decreased the intracellular transport (Fig. 7a, b, c).

DMT1 import of metal with a subsequent storage in ferritin has been previously demonstrated to be anti-oxidative (Ghio et al. 2005). While Zn^{2+} exposure was associated with increased DMT1 and ferritin and decreased oxidative stress, DMT1 did not appear to directly transport zinc. An alternative pathway by which zinc could affect the observed endpoints would be to change iron homeostasis so as to increase Fe^{2+} uptake. Incubations of BEAS-2B and human bronchial epithelial cells with both zinc sulfate and ferric ammonium citrate resulted in elevated intracellular concentrations of both metals (Fig. 8a, b).

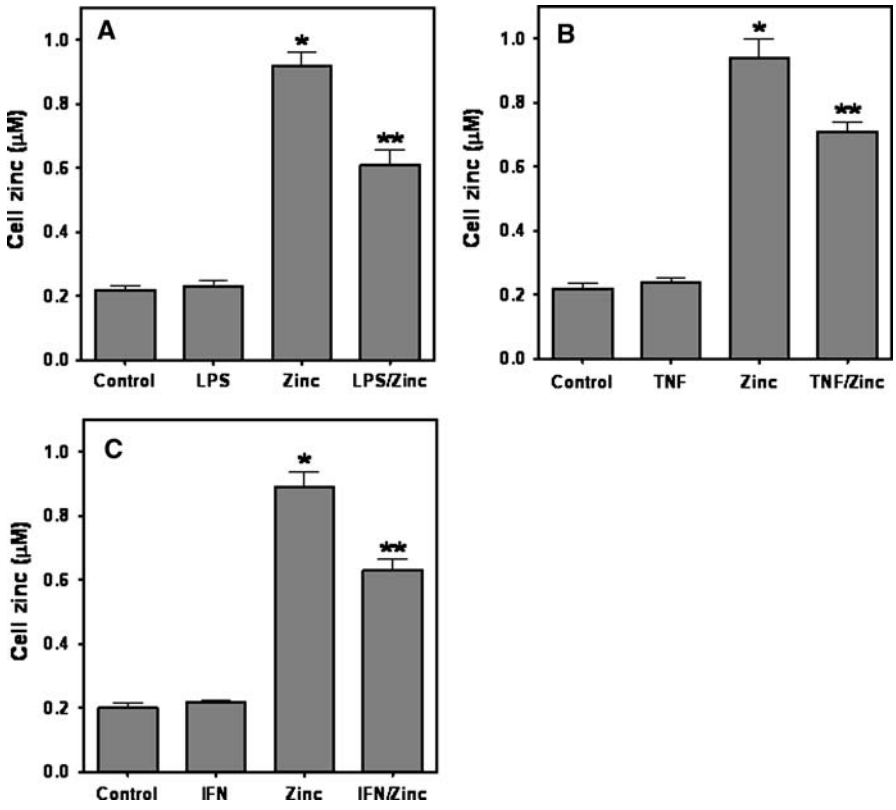

Fig. 7 Pre-incubation with endotoxin, TNF-α, and IFN-γ did not increase zinc uptake by BEAS-2B cells. Following 24 h of pre-treatment with 100 μg/ml endotoxin (**a**), 50 ng/ml TNFα (**b**), and 100 ng/ml IFN-γ (**c**) in KGM for 24 h, cells were treated with 25 μM Zn^{2+} in DPBS for 8 h, cells extracts were hydrolyzed and analyzed for zinc using ICPOES. $*P < 0.05$ compared with exposure to buffer only. $*P < 0.05$ compared with exposure to 25 μM $ZnSO_4$

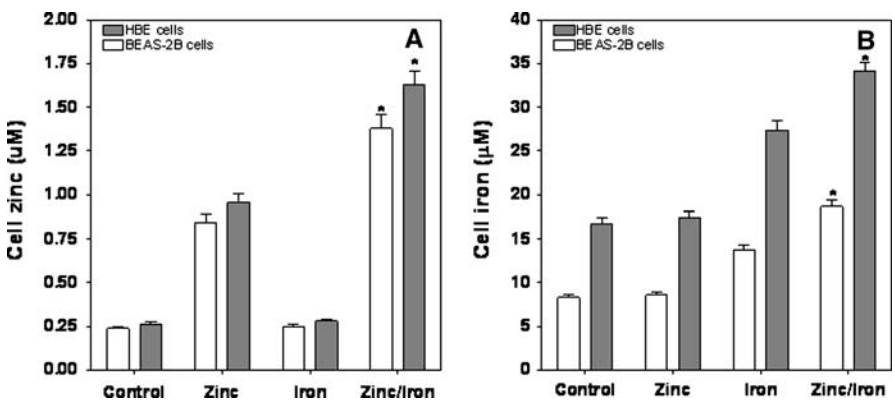

Fig. 8 Co-incubations of respiratory epithelial cells with zinc and iron increase uptake of both metals. BEAS-2B and human bronchial epithelial cells were exposed to 25 μM Zn^{2+} and 25 μM Fe^{3+} simultaneously for 8 h. Cellular zinc (**a**) and iron (**b**) were measured using ICPOES (**a**). The results shown are the mean ± standard deviation of a representative of three or more experiments. $*P < 0.05$ compared with exposure to buffer only

Discussion

Zinc plays critical roles as a co-factor in numerous transcription factors (including the ubiquitous zinc finger DNA binding proteins) and in a wide variety of biochemical processes and functions for over 300 different enzymes (Vallee and Auld 1990). Specific biological roles for zinc have been defined in regulation of paracrine function and neuronal activity through a mechanism of vesicle-mediated metal excretion and uptake. Subsequently, maintenance of intracellular zinc homeostasis is critical. This is

accomplished by import and export systems and storage of intracellular metal. Zinc uptake systems use integral membrane transport proteins to move the metal across the lipid bilayer of the plasma membrane. Such transport has been shown to be concentration-and time-dependent and saturable in cells not resident in the respiratory tract (Kambe et al. 2004). BEAS-2B and human bronchial epithelial cells similarly transported zinc intracellularly in both a concentration- and time-dependent manner. Following exposures to $ZnSO_4$ with subsequent intracellular transport, the metal was released into the extracellular media for up to 24 h. Such release by airway epithelial cells appeared time-dependent with a majority occurring in the initial 4 h. Comparable to other cell types, BEAS-2B and human bronchial epithelial cells appear to maintain intracellular zinc concentrations to support growth but do not accumulate excessive quantities therefore avoiding toxicity.

There are two families of zinc transporters: ZIP (ZRT, IRT-like protein) and CDF (cation diffusion facilitator) proteins. ZIP members facilitate zinc influx into the cytosol and are expressed in all organisms. There are at least 15 different ZIP-coding genes in humans and these proteins share the same basic structure (i.e., eight transmembrane segments and N- and C- termini facing the extracellular side of plasma membrane). ZIP1 and ZIP2, localized in the plasma membrane, allow cell uptake of zinc from the extracellular medium (Gaither and Eide 2000, 2001; Grotz et al. 2001). BEAS-2B cells had mRNA expression for both ZIP1 and ZIP2. However, there was no change in the quantity of ZIP1 and ZIP2 mRNA following exposure to elevated concentrations of zinc (25 μM Zn^{2+}). The absence of down-regulation of this mRNA with zinc exposure suggests the possibility of alternative pathways for this metal to move intracellularly.

Although zinc is essential, excess concentrations can be toxic to cells. While maintaining adequate levels of zinc for growth, cells must also control intracellular levels when exposed to excessive concentrations. Metallothionein proteins may participate in detoxification (Hamer 1986). ZIP transporters can also participate in detoxification by facilitating intracellular sequestration within organelles, or efflux of zinc across the plasma membrane. Finally, in multicellular organisms, cellular zinc efflux systems are required for the distribution of dietary zinc to other tissues. CDF members facilitate a release of zinc from the cytosol to an extracellular site. The CDF family (currently numbering eight in humans) was initially identified as a group of proteins conferring metal resistance to cells (Nies and Silver 1995). Increased expression of ZnT-1 and ZnT-2 can follow exposure to zinc (Langmade et al. 2000; McMahon and Cousins 1998) consistent with a possible role in detoxification (Kim et al. 2000; Palmiter et al. 1996). By increasing zinc efflux, ZnT-1 expression allows cell growth in the presence of elevated concentrations of extracellular metal (Palmiter and Findley 1995) and confers zinc resistance to wild-type cell lines (Kim et al. 2000; Palmiter and Findley 1995). ZnT-1 is expressed ubiquitously, localizes to the plasma membrane, and has been proposed as a zinc exporter out of the cells. BEAS-2B cells were comparable to other cell types with both increases in ZnT-1 mRNA (Langmade et al. 2000; Tsuda et al. 1997) and a lack of any change in ZnT-4 mRNA after zinc exposure (Liuzzi et al. 2001).

Zinc and iron deficiencies frequently occur together. The two metals can compete with each other for gastrointestinal absorption (O'Brien et al. 2000) and therefore interest in an interaction between zinc and iron transport has previously focused on antagonism between the two metals (Kordas and Stoltzfus 2004). The exchange between the two metals was initially attributed to competition for DMT1 transport by the enterocyte (Schultink et al. 1997). Investigation has previously demonstrated that zinc can either increase both DMT1 expression and cell Fe^{3+} uptake (Yamaji et al. 2001) or have no effect (Bannon et al. 2003; Tandy et al. 2000). Studies have also shown that DMT1 does not participate in Zn^{2+} transport by the enterocyte (Tandy et al. 2000; Tallkvist et al. 2000; Yamaji et al. 2001). While an affinity of DMT1 for Zn^{2+} is evident (Gunshin et al. 1997), transport by this protein does not appear to follow. In respiratory epithelial cells, DMT1 can be included in a metal detoxification pathway to control the oxidative stress presented by Fe^{3+} (Ghio et al. 2005). This metal transport by DMT1 culminates in the storage of metal in ferritin where it presents a diminished oxidative stress to the cell. Similar to at least one investigation of intestinal epithelial cells (Yamaji et al. 2001), zinc exposure in our study increased protein expression of

DMT1 by bronchial epithelial cells. In addition, ferritin concentration in BEAS-2B cells increased with zinc. Oxidative stress in these respiratory epithelial cells, measured as lipid peroxides, decreased with the zinc exposure. These results suggested that zinc exposure could increase DMT1 expression which then stored the metal and subsequently contained an associated oxidative stress. However, increased DMT1 expression with cytokine and endotoxin exposures did not affect zinc uptake; to the contrary, intracellular Zn^{2+} transport was decreased.

Alteration of iron homeostasis can affect both transport and biological effect exerted by several different metals (Kang et al. 2006; Martelli and Moulis 2004; Wu et al. 2006). Iron is an essential micronutrient utilized in almost every aspect of normal cell function. Cells have developed specific strategies to procure and utilize iron for cellular function. By changing the uptake, release, and storage of iron, another metal can subsequently exert a biological effect. Interactions between zinc and iron have been described and it is possible that some biological effects of zinc result from an impact on iron homeostasis. Co-incubations of these metals showed increased uptake of both zinc and iron by respiratory epithelial cells. This suggests that an effect of zinc in airway cells is to increase the uptake of iron. The specific pathway of increased transport was not identified but could involve numerous different avenues (e.g., calcium channels, ATPase, etc.). Elevated intracellular iron levels, the result of zinc mediated increases in iron transport. Through interactions with the iron responsive element/iron regulatory protein system (Leipuviene and Theil 2007), greater concentrations of intracellular iron would affect increased DMT1 and ferritin concentrations. Finally, these changes in protein expression could function to decrease oxidative stress in the cell.

We conclude that respiratory epithelial cells have a capacity to import and export zinc. DMT1 does not appear to transport zinc in these cells. However, zinc exposure increases iron uptake in this specific cell. The resultant elevations in cell iron may possibly affect an increased expression of DMT1 and ferritin; both proteins function to diminish oxidative stress. Comparable to determining the biological effects of other metals, the effect of zinc on iron transport in a specific cell type or a tissue must be considered.

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