Nickel Mobilizes Intracellular Zinc to Induce Metallothionein in Human Airway Epithelial Cells

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We recently reported that induction of metallothionein (MT) was critical in limiting nickel (Ni)-induced lung injury in intact mice. Nonetheless, the mechanism by which Ni induces MT expression is unclear. We hypothesized that the ability of Ni to mobilize zinc (Zn) may contribute to such regulation and therefore, we examined the mechanism for Ni-induced MT2A expression in human airway epithelial (BEAS-2B) cells. Ni induced MT2A transcript levels and protein expression by 4 hours. Ni also increased the activity of a metal response element (MRE) promoter luciferase reporter construct, suggesting that Ni induces MRE binding of the metal transcription factor (MTF-1). Exposure to Ni resulted in the nuclear translocation of MTF-1, and Ni failed to induce MT in mouse embryonic fibroblasts lacking MTF-1. As Zn is the only metal known to directly bind MTF-1, we then showed that Ni increased a labile pool of intracellular Zn in cells as revealed by fluorescence-activated cell sorter using the Zn-sensitive fluorophore, FluoZin-3. Ni-induced increases in MT2A mRNA and MRE-luciferase activity were sensitive to the Zn chelator, TPEN, supporting an important role for Zn in mediating the effect of Ni. Although neither the source of labile Zn nor the mechanism by which Ni liberates labile Zn was apparent, it was noteworthy that Ni increased intracellular reactive oxygen species (ROS). Although both *N*-acetyl cysteine (NAC) and ascorbic acid (AA) decreased Ni-induced increases in ROS, only NAC prevented Ni-induced increases in MT2A mRNA, suggesting a special role for interactions of Ni, thiols, and Zn release.

Keywords: nickel; metallothionein; zinc; epithelium

Nickel (Ni) is a well-known environmental and occupational hazard present in air pollution (1), cigarette smoke (2), diesel exhaust (3), and welding fumes (4, 5). Ni is a common component of alloy metals and is used in electroplating, stainless steel, coins, and jewelry (4, 6, 7). Inhalation of Ni has been associated with lung and nasal cancers (5, 8–10), fibrosis, and various other cardiopulmonary diseases (1, 5), including acute lung injury (4, 11).

Metallothioneins (MT) are highly conserved, small molecular weight, cysteine-rich proteins. In humans, the MT-gene family contains more than 10 members, of which MT2A is most commonly expressed (reviewed in Ref. 12). MT sequesters metals and thus maintains zinc (Zn) homeostasis and protects cells from metal toxicity (13–15). MT also appears to be important in limiting injury due to reactive oxygen and nitrogen species (16). MT transcripts increase during hyperoxic lung injury (17), in response to lipopolysaccharide (LPS) and diesel exhaust particles (18), and in ovalbumin-induced airway inflammation (19). Furthermore, MT transgenic mice are resistant to Ni- (20) and cadmium (Cd)-induced toxicity (12), whereas MT null mice are more susceptible

CLINICAL RELEVANCE

This study elucidates the mechanism of Ni-induced MT2A and provides a better understanding of how metals other than Zn induce this adaptive response. This understanding may offer insight for developing protective strategies to reduce pathogenic airway responses to inhaled metals.

to injury from exposure to Ni (20), Cd (12), or LPS (21). Thus, MT is protective against lung injury (22) and may even serve as a therapeutic target in airway diseases (19).

MT is induced by and capable of binding 18 different metals including Zn and Ni (reviewed in Ref. 23), but the precise mechanism of its induction is only known for Zn. Zn-induced MT2A is primarily transcriptionally regulated by Zn causing the metal transcription factor-1 (MTF-1) to bind multiple copies of the metal response element (MRE) in the promoter region (12, 24). The interaction of Zn with MTF-1 is unique since this Zn finger protein is directly activated only by Zn relative to other metals (25). Since MT plays an essential role in protecting the lung from Ni-induced injury, we investigated the hypothesis that Ni increases MT2A transcript levels in human bronchial airway epithelial cells by mobilizing Zn. These data support a role for intracellular Zn in the signaling pathway for Ni-induced increases in MT.

MATERIALS AND METHODS

Cell Culture

Human bronchial epithelial cells (BEAS-2B) (ATCC, Manassas, VA) were cultured in LHC-9 medium (Invitrogen, Carlsbad, CA) on tissue culture plates precoated with a matrix containing 0.01 mg/ml of human fibronectin (Invitrogen), 0.029 mg/ml Vitrogen 100 (COHESION Inc., Palo Alto, CA), and 0.01 mg/ml bovine serum albumin (Invitrogen) in LHC-9 medium. The cells were maintained at 37°C under an atmosphere of 5% CO₂ as described previously (26). Experiments were performed on 1-day postconfluent cells unless otherwise indicated. The medium was changed 12 to 16 hours before the experiment. Under these conditions, the BEAS-2B responses to metals are similar to responses in primary human bronchial epithelial cells grown in air/liquid interface cultures (27). Dko7 cells are SV-40 immortalized mouse embryonic fibroblasts (MEF) derived from MTF-1 double knockout embryonic stem cells (28). Wild-type MEFs and dko7 s were grown at 37°C under an atmosphere of 10% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Pittsburgh, PA), 4 mM L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The medium was changed 12 to 16 hours before the experiment to medium containing 0.1% bovine serum albumin (BSA) in replacement of FBS to arrest cell growth.

Treatments

NiSO₄ (Ni) and ZnCl₂ (Zn) solutions were prepared fresh from nickel(II) sulfate hexahydrate and zinc chloride (Sigma-Aldrich, St. Louis, MO), respectively. Cells were treated with 200 μM Ni and 100 μM Zn for the indicated times. In certain tests, cells were pretreated with 2 mM *N*-acetyl-L-cysteine (NAC) (Sigma) for 18 hours, 2 mM *L*-Ascorbic

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acid (AA) (Sigma) for 30 minutes, or 5 μ M *N,N,N',N'*-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Sigma) for 30 minutes.

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified by measuring absorbance (260 nm). Total RNA was reverse transcribed and the cDNA was amplified by real-time PCR (MJ Research Opticon 2; Bio-Rad Laboratories, Hercules, CA), as described previously (29). The following primers sets were used: MT2A (forward, 5'-CAACCTGTCCCGACTCTAGC-3'; reverse, 5'-TGGGAAGTCGC GTTCTTTACAT-3'); Ribosomal protein L13A (RPL13A) (forward, 5'-CGAGGTTGGCTGGAAGTACC-3'; reverse, 5'-ATTCCAGG GCAACAATGGAG) (gift from K. Brant [30]); Mt1 (forward, 5'-CA CCAGATCTCGGAATGGAC-3'; reverse, 5'-AGGAGCAGCAGC TCTTCTTG); β -actin (forward, 5'-GGGACCTGACCGACTACC TC-3'; reverse, 5'-GGGCGATGATCTTGATCTTC-3'). Gene expression was quantified using standard curves for the respective cDNA products. All changes in MT2A and Mt1 cDNA levels were normalized to RPL13A and β -actin transcript levels, respectively. Data are presented as mean $\pm$ SEM of fold change over control.

Transient Transfections and Luciferase Assays

Cells (70–80% confluence) were transfected with 1 μ g of a luciferase reporter construct driven by four MRE tandem repeats (pLucMRE; kind gift from D. Giedroc [31]) and 0.4 μ g *Renilla* luciferase reporter construct (pRL-TK; Promega, Madison, WI) using Lipofectamine and PLUS reagents (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Relative light units (RLU) were determined in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Results are expressed as a ratio of firefly luciferase activity to *Renilla* luciferase activity.

Total Protein Isolation and Western Blotting Analysis

Cells were rinsed twice in stop buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.1 M NaF, 0.2 M sucrose, 5 mM sodium pyrophosphate) supplemented with 100 μ M sodium orthovanadate and protease inhibitors and scraped in boiling lysis buffer (20 mM Tris, pH 7.5, 1% SDS). The lysates were boiled (5 min) and protein concentrations were determined by the absorbance (595 nm) after the addition of Coomassie blue dye (Thermo Fisher Scientific) using BSA as a reference standard. For Western blotting analysis of MT, 40 μ g of total cell lysate was incubated (37°C; 15 min) with *tris*(2-Carboxyethyl)phosphine (TCEP) (1 mM) followed by an incubation (37°C; 30 min) with iodoacetamide (20 mM). Proteins were separated on a 18% gel (Bio-Rad Laboratories) and transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was fixed in 2% glutaraldehyde (Sigma) and incubated with anti-MT antibody (Dako, Carpinteria, CA) overnight. Reacted bands were detected by anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ) and enhanced chemiluminescence substrates (Perkin Elmer, Inc., Wellesley, MA). Membranes were reprobed with β -actin to ensure equal loading.

MTF-1 Localization

BEAS-2B cells were transiently transfected with eGFP-MTF-1, a reporter molecule consisting of enhanced green fluorescent protein (eGFP) fused to the N-terminus of MTF-1 as described previously (32). Twenty-four hours after transfection, cells were trypsinized, replated on chamber well slides, and allowed to attach overnight. Cells were exposed to Ni (200 μ M; 30 min to 4 h) and Zn (100 μ M; 2 h). Cells were fixed with 4% paraformaldehyde (Sigma) and nuclei were stained with DRAQ5 (Biostatus Limited, Leicestershire, UK). Images were taken at $\times 60$ magnification with an Olympus Fluoview 500 confocal microscope in the University of Pittsburgh Center for Biological Imaging. Images were quantified by dividing the total number of eGFP-MTF-1-positive nuclei by the total number of nuclei in each image. Data are represented as mean $\pm$ SEM of the percentage of eGFP-MTF-1-positive nuclei.

Reactive Oxygen Species Measurements

Reactive oxygen species (ROS) were detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester

(CM-H₂DCFDA) (Invitrogen). Cells were seeded in a 24-well plate and preincubated with NAC or AA. Cells were incubated (37°C; 10 min) with CM-H₂DCFDA (20 μ M) and exposed to Ni (10 min). Fluorescence was read using a fluorescent plate reader (excitation [485 nm] emission [530 nm]).

Flow Cytometry

Cells were incubated (37°C; 1 h) with 5 μ M free zinc fluorophore FluoZin-3 AM ester (Invitrogen) with Pluronic F-127 (equal volume) (Invitrogen) in HBSS containing calcium and magnesium (Invitrogen). Cells were rinsed with HBSS and treated (30 min) with the ionophore, 2-mercaptopyridine-*N*-oxide sodium salt (pyrithione) (Sigma) in the presence or absence of either 20 μ M Ni or 10 μ M Zn. FluoZin-3 can compartmentalize in the cell after long exposure times and also becomes cytotoxic. To circumvent this, pyrithione was added to enable the metals to enter the cell more readily without relying on transporters. The concentrations of metals used were reduced by 10-fold to avoid toxicity. TPEN (50 μ M) was added (5 min) to chelate the free zinc. Cells were rinsed in PBS, trypsinized, and centrifuged at 1,000 $\times$ g for 10 minutes. The pellet was resuspended with PBS containing 100 μ g/ml propidium iodide and incubated (37°C; 1 h) in the dark to stain dead cells. Flow cytometry was performed using a FACSCanto (BD Biosciences, San Jose, CA). The histograms are representative of three separate experiments. The percentage of negative and positive FluoZin-3 cells is shown in the boxes on the left and right sides of the gated populations, respectively (Figure 6A).

Statistics

One-way ANOVA was used to determine whether the mean of each treatment was different from the untreated cells (control). Dunnett's or Tukey's Multiple Comparisons *post hoc* tests were used to determine significant differences between the means of each group. Linear trend analysis was performed on Figure 1A to determine if there was a time-dependent response. Two-way ANOVA with a Dunnett's Multiple Comparison *post hoc* test was performed on Figure 3C to determine if there was a difference between the two cell lines after the Ni exposure. All statistics were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). Data are represented as mean $\pm$ SEM or as fold control.

RESULTS

Ni Increases MT Expression and MRE Transactivation

Exposure to Ni is known to induce MT expression in hepatocytes (13) and in mouse lung (33). To examine the effects of Ni on MT2A mRNA levels in airway epithelial cells, BEAS-2B cells were exposed to 200 μ M Ni (up to 48 h). Ni increased MT2A transcript levels significantly by 2 hours and remained elevated at 24 hours, with maximal induction occurring at 4 hours after exposure (Figure 1A). This induction was time-dependent as determined by linear trend analysis. Moreover, Ni increased MT protein after 4 hours, as shown by Western blotting (Figure 1C). It is well understood that MT expression is regulated at the level of transcription (24). Although there are numerous cis-elements in the promoter region of MT2A, MTF-1 transactivation of MRE is essential for both basal and inducible MT expression (24). Therefore, we tested the hypothesis that Ni stimulates the transactivation of MREs to induce MT2A mRNA levels. pLucMRE luciferase activity increased in cells exposed to 200 μ M Ni for 4 hours or 8 hours (Figure 1B). These data suggest that the induction of MT2A by Ni is transcriptionally regulated.

Nickel Stimulates the Transactivation of MRE and Induces MT2A through a Zn-Dependent Pathway

Zn increases the binding activity of MTF-1 to induce MT2A (14), but the mechanism for other metals, especially Ni, is poorly resolved. Therefore, we exposed the cells to Ni in the presence or absence of TPEN, a Zn chelator, to examine whether Ni activates

Figure 1. Ni increases metallothionein (MT) expression and metal response element (MRE) transactivation. (A) BEAS-2B cells were exposed to 200 μ M Ni for the indicated times and total RNA was isolated. MT2A mRNA levels were measured by real-time PCR and were normalized to the housekeeping gene RPL13A. Data represent mean $\pm$ SEM of fold control ($n = 3$). (B) BEAS-2B cells were transiently transfected with pLucMRE. Twenty-four hours after transfection, cells were exposed to 200 μ M Ni for the indicated times. Cells were lysed and luciferase assays were performed. Data represent mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with untreated cells. (C) BEAS-2B cells were left untreated or exposed to Ni for 4 hours. Total protein was isolated and western analysis for MT was performed. The data are representative of duplicate samples from three separate experiments.

MREs to induce MT2A transcripts by increasing free intracellular Zn. Cells that were transiently transfected with pLucMRE and exposed to TPEN and Ni had decreased luciferase expression compared with cells exposed to Ni alone (Figure 2A). Likewise, the data in Figure 2B show that TPEN pretreatment inhibited both Ni and Zn-induced MT2A mRNA levels. These data indicated that the Ni induction of MT2A required free intracellular zinc.

Ni Activates MTF-1 to Induce MT

To examine the effects of Ni on MTF-1 nuclear localization, we transiently transfected BEAS-2B cells with eGFP-MTF-1, a reporter molecule with eGFP fused to MTF-1 (32). After 24 hours, cells were left untreated or exposed to Ni from 1 to 4 hours. Exposure to Ni increased MTF-1 protein in the nucleus (Figures 3A and 3B). The requirement of MTF-1 for Ni-induced MT expression was also demonstrated using mouse embryonic fibroblast deficient in MTF-1 (dko7). Wild-type and dko7 cells were exposed to Ni for up to 4 hours and Mt1 transcript levels were measured. The data in Figure 3C show that in the absence of MTF-1, Ni fails to induce Mt1 transcripts, indicating that MTF-1 is essential in Ni stimulation of MT.

Ni Increases Intracellular ROS, but Ni-Induced MT2A Expression Does Not Require Intracellular ROS Production

We previously demonstrated that particulate Ni increases intracellular oxidant production using the fluorescent intracellular

Figure 2. *N,N,N',N'*-Tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) prevents Ni-induced MRE transactivation and MT2A mRNA expression. (A) BEAS-2B cells were transiently transfected with pLucMRE. Twenty-four hours after transfection, cells were pretreated with 5 μ M TPEN for 30 minutes before exposure to 200 μ M Ni for 4 hours. Cells were lysed and luciferase assays were performed. Data represent mean $\pm$ SEM ($n = 3$). (B) BEAS-2B cells were pretreated with 5 μ M TPEN before exposure to 200 μ M Ni or 100 μ M Zn for 2 hours. Total RNA was isolated and MT2A mRNA levels were measured by real-time PCR and normalized to the housekeeping gene RPL13A. Data represent mean $\pm$ SEM of fold control ($n = 3$). * $P < 0.05$ and *** $P < 0.001$ compared with untreated cells; ^^^ $P < 0.001$ compared with cells treated with Ni alone; ~~~~ $P < 0.001$ compared with cells treated with Zn alone.

Figure 3. Ni activates MTF-1 to induce MT expression. (A) BEAS-2B cells were transiently transfected with eGFP-MTF-1. Twenty-four hours after transfection, cells were replated on chamber slides and exposed to 200 μ M Ni for 1 to 4 hours or 100 μ M Zn for 2 hours. Cells were fixed with 4% paraformaldehyde and nuclei were stained with DRAQ5. The representative images were captured at $\times 60$ with an Olympus Fluoview 500 confocal microscope. (B) Quantitative analysis of eGFP-MTF-1-positive nuclei is presented as mean $\pm$ SEM. * $P < 0.05$ compared with untreated cells. (C) WT and dko7 cells were exposed to 200 μ M Ni for the indicated times. Total RNA was isolated and Mt1 mRNA levels were measured by real-time PCR and normalized to the housekeeping gene β -actin. Data represent mean $\pm$ SEM of

fold control ($n = 3$). ** $P < 0.01$ and *** $P < 0.001$ compared with untreated cells (0 h). ^^ $P < 0.01$ and ^^ $P < 0.001$ compared with wild-type MEF cells (WT) at the corresponding time.

oxidant indicator CM-H₂DCFDA in BEAS-2B cells (34). The data in Figure 4 demonstrated that soluble Ni also increases intracellular ROS in BEAS-2B cells. Increasing the intracellular reduced thiol pool by incubating the cells with 2 mM NAC for 18 hours or increasing the levels of the antioxidant by incubating the cells with 2 mM ascorbic acid for 30 minutes inhibited Ni stimulated ROS production. However, only treatment with NAC blocked Ni-induced increases in MT2A transcript levels; suggesting that MT2A is induced by Ni through a thiol-dependent, but not ROS-dependent pathway (Figure 5).

Nickel Increases Intracellular Free Zinc Levels

To investigate whether Ni signals by increasing free intracellular Zn levels, we performed flow cytometry on live cells loaded with the Zn-sensitive fluorophore FluoZin-3. The data in Figure 6A demonstrated that exposure to Ni or Zn increased FluoZin-3 fluorescence from 0.2% to 42.1% and 93.8% of total cells, respectively. The addition of TPEN attenuated the Ni-induced

increase in FluoZin-3 (42.1% to 12.0% of total cells) fluorescence, suggesting that Ni mobilized intracellular Zn. These experiments used the ionophore, pyrithione, to facilitate the entry of Zn and Ni into the cells. We found that exposure to 20 μ M or 200 μ M Ni in the presence of ionophore mobilized the

Figure 4. Ni-induced intracellular ROS production is prevented by antioxidants. BEAS-2B cells were pretreated with 2 mM NAC for 18 hours or 2 mM L-Ascorbic acid (AA) for 30 minutes before loading cells with 20 μ M CM-H₂DCFDA for 10 minutes. Cells were then exposed to 200 μ M Ni for 10 minutes. Fluorescence was measured with a fluorescent plate reader with excitation at 485 nm and emission at 530 nm. Data represent mean $\pm$ SEM ($n = 3$). *** $P < 0.001$ compared with untreated cells; ^^ $P < 0.001$ compared with cells treated with Ni alone.

Figure 5. NAC, but not AA, prevents Ni-induced MT2A mRNA levels. BEAS-2B cells were pretreated with (A) 2 mM NAC for 18 hours or (B) 2 mM L-Ascorbic acid (AA) for 30 minutes before exposure to 200 μ M Ni for 2 hours. Total RNA was isolated and MT2A mRNA levels were measured by real-time PCR and were normalized to the housekeeping gene RPL13A. Data represent mean $\pm$ SEM of fold control ($n = 3$). ** $P < 0.01$ and *** $P < 0.001$ compared with untreated cells; ^^ $P < 0.001$ compared with cells treated with Ni alone.

Figure 6. Ni increases free intracellular zinc. (A) BEAS-2B cells were loaded with 5 μ M FluoZin-3 AM ester with Pluronic F-127. Cells were exposed to 20 μ M Ni or 10 μ M Zn in the presence of 5 μ M pyrithione for 30 minutes. TPEN was added for 5 minutes before harvesting to chelate the Zn. FluoZin-3 fluorescence was measured by flow cytometry. Data represent the results of three separate experiments. The percentages on the left and right sides of the histograms represent negative and positive FluoZin-3 fluorescence, respectively. (B) BEAS-2B cells were exposed to 20 μ M Ni in combination with 5 μ M pyrithione or 200 μ M Ni alone. Total RNA was isolated and MT2A mRNA levels were measured by real-time PCR and were normalized to the housekeeping gene RPL13A. Data represent means $\pm$ SEM of fold control ($n = 3$). *** $P < 0.001$ compared with untreated cells.

same amount of labile zinc. The data in Figure 6B demonstrate that exposure to 20 μ M Ni in combination with 5 μ M pyrithione had a similar induction of MT2A mRNA levels after 4 hours compared with 200 μ M Ni without ionophore.

DISCUSSION

Ni is a major component of ambient air particulate matter (1, 35) that is associated with inappropriate immune responses (35, 36), airway hyperresponsiveness (7, 37), and increased cardiopulmonary disease (1). Mechanisms of action for inhaled Ni that are independent of its antigenicity remain poorly defined. It is evident, however, that without induction of protective or

adaptive genes, such as increasing MT in airway epithelium, the pathogenic responses to inhaled Ni are greatly enhanced (20, 33). These studies used the BEAS-2B cells to model the terminal bronchiolar epithelial responses to Ni. While these cells are SV40 immortalized, we have demonstrated that their responses to metals are nearly identical to primary normal human bronchiolar epithelial cells cultured on an air/liquid interface (27) and mouse bronchiolar epithelium *in vivo* (29). Induction of MT after Ni exposure in these cells closely matches responses observed in mouse lungs (33), and thus the mechanisms for this induction in BEAS-2B cells are likely to be broadly applicable. As indicated, MT induction by Ni and other toxic metals is a protective response. However, since only Zn

has been shown to directly induce the gene, it remains unclear how Ni and other metals signal for indirect gene activation. The current studies indicate that Ni transcriptionally activates MT2A by first mobilizing intracellular Zn to bind with MTF-1. Ni does not elicit ROS generation to mobilize labile Zn pools, but may displace Zn from thiols or interact with regulatory reduced thiols to stimulate MTF-1 transactivation.

MT regulates zinc homeostasis and is induced by various stressors, including metals (13–15). We demonstrated that Ni exacerbated acute lung injury in MT null mice (20), acknowledging its protective function in the lung. MT is basally expressed in the airway epithelium (38) and inducible by exogenous chemicals (39, 40). Our data in Figure 1A corroborate previous findings that Ni induces MT2A mRNA (13, 33). This induction was rapid and time-dependent, with the highest expression after 4 hours of Ni exposure. Ni also increased MT protein levels after 4 hours of Ni exposure (Figure 1C). There are multiple transcription factors that drive MT2A gene expression, but the activation of MTF-1 has an essential role by binding to MREs present in the promoter region of MT2A (24). Ni increased transactivation of MRE (Figure 1B), which occurred through the increased binding activity of MTF-1. Exposure to Ni facilitated the translocation of MTF-1 to the nucleus (Figures 3A and 3B). Also, Ni did not induce MT in cells deficient in MTF-1 (Figure 3C). However, since only Zn can directly activate and increase DNA binding of MTF-1 (24, 25), the data support the hypothesis that Ni-induced MT2A is caused by redistributed Zn stimulating MTF-1 DNA binding.

Soluble Ni increased intracellular ROS (Figure 4), which is consistent with our previous report using particulate Ni₃S₂ (34). Likewise, NAC and AA prevented Ni-induced intracellular ROS levels. However, in the previous report, neither antioxidant affected transcriptional induction of SERPINE1 after Ni exposures (34). In contrast, in the current study, only NAC prevented Ni-induced MT2A transcript levels (Figure 5). Thus, production of ROS may not be involved in Ni-induced MT2A, but free thiols may regulate the ability of Ni to induce certain genes. NAC acts as an antioxidant by increasing the intracellular free thiol pool and increasing synthesis of glutathione (GSH) (41). GSH and oxidized glutathione (GSSG) are key regulators of the release of Zn from MT (42, 43). GSSG interacts with MT to facilitate the transfer of Zn from MT (43). However, in reducing conditions, when GSH concentrations are high, Zn remains bound to MT (42). The data from Figure 5 suggest that adding NAC pushed the thiol balance toward enhanced Zn binding to MT and prevented its mobilization to interact with MTF-1. Exposure to high levels of Ni has been shown to reduce the intracellular GSH content and increase the GSSG/GSH ratio (44, 45). Hence, it is possible that in our system Ni decreased the GSH content of the cell to facilitate Zn mobilization. However, this is unlikely given the low level of oxidant generation in response to Ni and the overwhelming amount of intracellular GSH relative to the amount of Ni added.

Transcriptional activation of MT2A by Ni required Zn mobilization, since pretreatment with TPEN, a Zn chelator, abrogated Ni-induced MRE transactivation and increased MT2A transcripts (Figure 2). Although TPEN is often used as a Zn-specific chelator, it has affinities for other divalent metals including Ni (15). However, the stoichiometry of TPEN metal chelation is such that the low concentration used in the present study was sufficient to bind only 5% of the added Ni. Furthermore, the ability of Zn, but not Ni, to rescue the response observed (Figure 2B) indicates that the added TPEN predominantly bound the intracellular Zn released by Ni to inhibit Ni-stimulated MT induction. This inhibition did not result from

toxicity, since TPEN was added at a concentration that did not cause cell death (data not shown). Thus, the observed results consistently support a role for mobilization of endogenous Zn in Ni-stimulated signaling. Moreover, increased FluoZin-3 fluorescence after Ni exposure (Figure 6) established that Ni increases free intracellular Zn levels, as was previously demonstrated in hepatocytes (46).

In summary, we demonstrated that Ni increases free intracellular Zn levels to induce MT2A transcript levels. This was not at the level of MT itself, since Ni has a low affinity for MT, relative to Zn, and cannot displace Zn from MT (15). The precise mechanism of how Ni is redistributing Zn has not been investigated fully, but remains an area of interest. However, this study elucidates the mechanism of Ni-induced MT2A and provides a better understanding of how metals other than Zn produce this adaptive response. This understanding may offer insight for developing protective strategies to reduce pathogenic airway responses to inhaled metals.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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