Divalent metal transporter-1 decreases metal-related injury in the lung

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Ghio, Andrew J., Claude A. Piantadosi, Xinchao Wang, Lisa A. Dailey, Jacqueline D. Stonehuerner, Michael C. Madden, Funmei Yang, Kevin G. Dolan, Michael D. Garrick, and Laura M. Garrick. Divalent metal transporter-1 decreases metal-related injury in the lung. Am J Physiol Lung Cell Mol Physiol 289:L460–L467, 2005. First published May 20, 2005; doi:10.1152/ajplung.00154.2005.—Exposure to airborne particulates makes the detoxification of metals a continuous challenge for the lungs. Based on the fate of iron in airway epithelial cells, we postulated that divalent metal transporter-1 (DMT1) participates in detoxification of metal associated with air pollution particles. Homozygous Belgrade rats, which are functionally deficient in DMT1, exhibited diminished metal transport from the lower respiratory tract and greater lung injury than control littermates when exposed to oil fly ash. Preexposure of normal rats to iron in vivo increased expression of the isoform of DMT1 protein that lacked an iron-response element (−IRE), accelerated metal transport out of the lung, and decreased injury after particle exposure. In contrast, normal rats preexposed to vanadium showed less expression of the +IRE isoform of DMT1, decreased metal transport, and greater pulmonary injury after particle instillation. Respiratory epithelial cells in culture gave similar results. Also, DMT1 mRNA and protein expression for the −IRE isoform increased or decreased in these cells when exposed to iron or vanadium, respectively. These results thus demonstrate for the first time a primary role for DMT1 in lung metal transport and detoxification.

THE PRESENCE OF A ROBUST surface clearance system in the mammalian lung appears to be necessary both to detoxify metals that provoke oxidative stress by generation of reactive oxygen species (ROS) and to defend the host against microbes that require iron for growth. Catalytically active metal, such as that associated with air pollution particles, causes lung injury (6) mediated by oxidant generation (11).

Natural resistance-associated macrophage protein 1 (Nramp1) is the prototype for a group of structurally and functionally related metal cation transporters in vertebrates. These proteins are well conserved across species with homologs identified in yeasts, bacteria, worms, flies, and plants, suggesting that they perform an important common function. Nramp2, now more frequently referred to as divalent metal transporter-1 (DMT1, but also DCT1 and SLC11A2), is expressed in most tissues and cell types as an integral membrane protein (26). This protein transports divalent metal cations including Fe2+ (9).

DMT1 generates two alternatively spliced mRNAs (4, 9) that differ at their 3′-untranslated region by the presence or absence of a classic iron-response element (IRE) (thus +IRE or −IRE, respectively). The corresponding protein isoforms also differ in their COOH termini in the last 18 or 25 amino acids. The IRE could allow DMT1 expression to be modulated by iron at the posttranscriptional level. In the lung, however, there is an IRE-independent iron-regulatory pathway for control of DMT1 expression, as exposure of respiratory epithelial cells to iron greatly increases expression of the −IRE isoform of DMT1, whereas the +IRE isoform shows little response to the metal (28). Additional isoforms of DMT1 occur due probably to alternative start sites for transcription (10), but expression and regulation of these isoforms remain to be studied in airway tissue.

The capacity of DMT1 to transport metal and its ubiquitous expression make it a candidate for transferrin-independent iron uptake. This mode of transport can result in the sequestration of iron in ferritin, where storage would diminish iron-induced ROS (6). Alternatively, divalent metal ion uptake by DMT1 could be counterproductive and increase damage to cells and tissue. In this paper, we demonstrate that DMT1 is essential for the transport and detoxification of some metals associated with an air pollution particle that damages the pulmonary epithelial surface.

MATERIALS AND METHODS

Materials. All reagents were from Sigma (St. Louis, MO) unless otherwise specified. The particle employed in these studies was an oil fly ash collected by Southern Research Institute (Birmingham, AL). This ash was emitted from the cyclone of a power plant in Florida that was burning a low-sulfur number 6 residual oil (collection temperature 250–300°C). It has been previously chemically characterized (11); there is little carbonaceous material in the particle, but it contains high concentrations of metals (11).

Exposure of animals to metal compounds and oil fly ash. The State University of New York-Buffalo Institutional and United States Environmental Protection Agency Animal Care and Use Committees reviewed and approved all procedures on Belgrade and Sprague-Dawley rats, respectively. Efforts were made to limit discomfort in the animals and to reduce the number of animals used in the experiments. We used Belgrade rats as a key to the role of DMT1 because they are functionally deficient in the transporter due to a G185R mutation (4).

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The rats were N8 generation b/b crossed into a Fischer 344 background (2). To improve husbandry, we maintained the colony on an iron-supplemented rat chow (PMI 5001 with 130 ppm FeSO4 added) (5). The controls were N8 +/+ littermates. After Belgrade and control rats were anesthetized with 2–5% halothane (Aldrich Chemicals, Milwaukee, WI), their lungs were instilled with either 0.5 ml of saline or 500 μg of oil fly ash in 0.5 ml of saline (n = 6/exposure). Twenty-four hours later, tissue iron and vanadium concentrations were measured in lung digests (3 N HCl/10% trichloroacetic acid) (23) using inductively coupled plasma atomic emission spectroscopy (ICPAES, model P30; Perkin Elmer, Norwalk, CT) at wavelengths of 238.204 and 292.402 nm for iron and vanadium, respectively. A second group of rats was instilled with either 0.5 ml of saline or 500 μg of oil fly ash in 0.5 ml of saline (n = 6/exposure). After another 24 h, the rats were euthanized under anesthesia and bronchoalveolar lavage (BAL) was performed using volumes of saline of 90% of total lung capacity determined from allometric equations (35 ml/kg body wt) (21). Saline was withdrawn after a 3-s pause, re-injected twice more, then recovered, and stored on ice. BAL neutrophils were enumerated by counting 200 cells per sample on slides stained with a modified Wright’s stain (Diff-Quick stain; American Scientific Products, McGaw Park, IL). After centrifugation of BAL at 600 g for 10 min to remove cells, soluble protein was determined in the supernatant with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard. Lactate dehydrogenase (LDH) concentration in BAL supernatant was also measured using a commercial kit (Sigma) as modified for automated measurement (Cobas Fara II centrifugal analyzer; Roche Diagnostic, Montclair, NJ).

Sixty-day-old male Sprague-Dawley rats were instilled instracheally with either 0.5 ml saline, 0.5 ml 100 μM ferric ammonium citrate (FAC), or 0.5 ml 10 μM vanadyl sulfate (VOSO4). Twenty-four hours later, the animals were again anesthetized and instilled with either 0.5 ml of saline or 500 μg of oil fly ash in 0.5 ml of saline (n = 8/exposure). After 15, 30, or 60 min, or 24 h (n = 4 per exposure per time point), the rats were euthanized and the lungs were excised and digested (3 N HCl/10% trichloroacetic acid) (23). Tissue nonHEME iron and vanadium concentrations were measured in the lung digests by ICPAES.

We tested the association between metal transport and lung injury by repeating the exposures above in separate rats (n = 8/exposure). Twenty-four hours after the exposures, the animals were again anesthetized and instilled with either 0.5 ml of saline or 500 μg of oil fly ash in 0.5 ml of saline (n = 4/exposure), and BAL was performed 24 h later. The BAL was used to determine differential cell counts and protein and LDH concentrations.

Lung immunohistochemistry. Sixty-day-old (250–300 g) male Sprague-Dawley rats were anesthetized and intratracheally instilled with 0.5 ml normal saline, 0.5 ml 100 μM FAC, or 0.5 ml 10 μM VOSO4. Twenty-four hours later, the animals were anesthetized and exsanguinated. The lungs were removed en bloc and fixed at inflation volume (5). The controls were N8 littermates. After Belgrade and control groups were instilled with 0.5 ml normal saline, 0.5 ml 100 μM FAC, or 50 μM VOSO4 or oil fly ash for time periods up to 1 h. Then supernatants were collected by centrifuging at 600 g × 10 min. The concentration of iron and vanadium in the supernatant was determined by ICPAES. Single element standards were used to calibrate the instrument (Fisher, Pittsburgh, PA).

Cellular uptake of iron and vanadium. After preexposures to FAC, medium, or VOSO4, cells were again exposed to either 100 μM FAC and 50 μM VOSO4 or oil fly ash for times up to 4 h. Then supernatants were collected by centrifuging at 600 g × 10 min. The concentration of iron and vanadium in the supernatant was determined by ICPAES. Simple element standards were used to calibrate the instrument (Fisher, Pittsburgh, PA). Lipid peroxides in cells exposed to oil fly ash. After preexposures to medium, FAC, or VOSO4, BEAS-2B cells were exposed to either medium or 100 μM FAC and 50 μM VOSO4 or oil fly ash for 4 h. The supernatant was removed. The cells were washed with PBS three times, scraped into 1.0 ml of 2,4-dinitrophenylhydrazine (DNPH) solution (0.125% in acetone), and vortexed. Dinitrophenylhydrazones were extracted as previously detailed and analyzed by HPLC-UV-mass spectrometry as described (13).

Statistics. Grouped data are expressed as mean values ± SE. Differences between groups were evaluated by one-way analysis of variance. Post hoc comparison was done by Scheffé’s test. Significance was assumed at P < 0.05. All studies were replicated twice except those involving Belgrade rats, which were not repeated to minimize cost and the use of a precious resource.

RESULTS

Metal transport and injury in lungs of the Belgrade rat. Respiratory epithelial cells appear to detoxify metal through sequential 1) uptake, 2) storage in ferritin, and 3) export in a catalytically less reactive state (31). Prior studies (6, 28) led to the hypothesis that −IRE DMT1 participated in uptake for this series of reactions. This hypothesis received support from increased −IRE DMT1 in the lungs of hypotransferrinemic mice, which was associated with greater resistance to metal damage (8). The Belgrade rat has a mutation G185R in DMT1
that inactivates the metal ion transporter (4). Homozygous b/b animals have a severe microcytic, hypochromic anemia due to poor iron absorption (5, 16). Heterozygous +/b rats appear normal in all ways thus far examined. If DMT1 activity protects against particle injury by transporting iron into airway cells where it can be sequestered or exported, then protection would fail in this animal model because of defective DMT1. Alternatively, if increased uptake were toxic, b/b rats would be better protected than +/b controls, whereas there would be no effect if the rise in −IRE DMT1 were irrelevant. Belgrade rats demonstrated less ability to remove iron from the lower respiratory tract than +/b littermates (Fig. 1A). Basal levels of iron remaining in the tissue reflect this metal’s essential role in lung epithelial cells (28), but decreased loss in the b/b lung demonstrates that DMT1 is normally responsible for at least some iron uptake into pneumocytes, and defective DMT1 transports less iron. These data also imply that removal necessarily depends on uptake although the data do not exclude a role for DMT1 in steps subsequent to epithelial uptake. Twenty-four hours after oil fly ash instillation, lung vanadium concentrations remained elevated in the Belgrade rats relative to control animals exposed to the same particle (Fig. 1B). This difference suggests but does not prove that VO²⁺ is also transported by DMT1. After particle instillation, the b/b rats also demonstrated significantly increased lung injury relative to +/b littermates (Fig. 1, C and D). There was no difference in the percentage of neutrophils in the BAL. Therefore, the metal ion transporter DMT1 normally functions in the lung to help remove divalent metal ions from the alveolar spaces. This uptake, at least for Fe, lessens damage as represented by lavage protein and LDH concentrations.

DMT1 immunohistochemistry, metal transport, and injury in lungs of normal rats. Figure 2 shows immunohistochemistry done after instilling saline, FAC, and VOSO₄ into the lungs of Sprague-Dawley rats. Relative to saline (Fig. 2A), FAC increased and VOSO₄ decreased staining for −IRE DMT1 (Fig. 2, B and C), whereas these same exposures had no effect on +IRE DMT1 (not shown). Iron exposure elevated −IRE DMT1 expression in macrophages and airway and alveolar epithelial cells. Vanadium appeared nearly to eliminate staining by the antibody in the epithelium, but alveolar macrophages still demonstrated some minimal staining.

Fig. 1. Lung iron and vanadium and injury in b/b rats and +/b littermates after instillation with oil fly ash. Animals were instilled with either 0.5 ml of saline or 500 μg of oil fly ash in 0.5 ml of saline. After 24 h, lung iron and vanadium concentrations were determined. Lung iron (A) and vanadium (B) concentration are increased in the b/b rats relative to the +/b animals at 24 h after exposure to the particle (vanadium was not measurable in any animal instilled with saline only). Relative to control +/b littermates, b/b rats also exhibited greater lung injury after oil fly ash as reflected by increased concentrations of protein (C) and LDH (D). There were no differences in the percentage of neutrophils (data not shown). *Significant difference compared with control animals; n = 6/group.
The lungs of Sprague-Dawley rats were preexposed to either saline, FAC, or VOSO₄ to leave unchanged, increase, or decrease the amount of −IRE DMT1, respectively. Twenty-four hours later the lungs were instilled with either saline or the particle. Lung vanadium concentration and injury were measured. In animals preexposed to FAC and then exposed to the particle, there was significantly greater metal clearance from the lung compared with saline pretreatment (Fig. 3A). Vanadyl sulfate pretreatment evoked the opposite effect; that is, there was significantly less metal clearance from the lungs of animals preexposed to vanadium and then treated with the particle (Fig. 3A). No vanadium was measurable in lung tissue collected at 24 h from any animal regardless of pretreatment. To determine lung injury, protein and LDH concentrations were measured in BAL. Preexposure to iron diminished lung injury 24 h after particle instillation (Fig. 3, B and C), whereas preexposure to vanadium increased lung injury after oil fly ash instillation (Fig. 3, B and C). Neither pretreatment affected neutrophil influx after particle exposure.

**RT-PCR and Western blots for DMT1 in BEAS-2B cells.** BEAS-2B cells were exposed to iron or vanadium to compare the effects of the two metals that are found in oil fly ash. FAC increased (Fig. 4A), whereas VOSO₄ decreased (Fig. 4B), mRNA for −IRE DMT1 in BEAS-2B cells. An effect of these exposures on +IRE DMT1 was not evident. Protein expression of −IRE DMT1 increased after iron (Fig. 4C) and decreased after vanadium exposures (Fig. 4D). The protein expression of +IRE DMT1 remained unaffected by any treatment (data not shown). Thus the amounts of both mRNA and protein for the −IRE isoform of DMT1 are affected by metal, increasing with iron exposure and decreasing with VOSO₄ exposure.

**DMT1 expression, metal transport, and oxidative stress in BEAS-2B cells.** To evaluate how changes in −IRE DMT1 expression correlate with functional changes, BEAS-2B cells were preexposed to medium, FAC, or VOSO₄ for 4 h to induce changes in the level of −IRE DMT1. The cells were then incubated again with FAC or VOSO₄ to investigate metal transport and oxidative stress. Intracellular metal transport was quantified by measuring supernatant concentrations; this methodology has been previously verified to reflect uptake rather than any precipitation or membrane associations of metal species (28). Uptake of iron increased after preexposure to iron and decreased after preexposure to VOSO₄ (Fig. 5A). Similarly, vanadium uptake by the respiratory epithelial cells exhibited parallel changes (Fig. 5B). We also investigated how the same preexposures affected the capacity of the cells to transport the same metals derived from oil fly ash. Preexposure to FAC again increased uptake of both iron and vanadium (Fig. 5, C and D), whereas preexposure to VOSO₄ still had the opposite effect and diminished metal uptake (Fig. 5, C and D). Thus uptake of iron or VOSO₄ from defined medium or oil fly ash, paralleled the changes in mRNA and protein for the −IRE isoform of DMT1.

Effects of increased metal transport on the cells were then evaluated with respect to oxidative stress. Exposure of BEAS-2B cells to the oil fly ash increased oxidative stress shown by elevations in acetaldehyde (Table 1). This index of ROS was decreased with FAC pretreatment and increased with VOSO₄ pretreatment. The results suggest a transport of metal by DMT1 that ultimately results in a less catalytically active state.

**DISCUSSION**

Previously, it could have been argued that the chain of events described here (iron exposure increasing −IRE DMT1 expression leading to metal uptake with sequestration of iron and control of the potential ROS) is just a set of associations. It was possible that increased expression actually and counterproductively potentiated oxidative stress or that the elevation
was irrelevant. The data on b/b rats, however, rule out these alternatives and support the argument that this chain of events is a set of causal relationships because b/b homozygotes have defective DMT1. The G185R mutation (G216R in isoforms containing exon 1A-7) diminishes transport activity (4). This transport deficiency in the Belgrade rat renders this animal ineffective at controlling the oxidative stress presented by the particle, so that greater tissue injury results. Although there is room for other explanatory hypotheses that connect the injury to the defective DMT1, one can no longer maintain that higher DMT1 activity places cells at higher risk of damage.

As iron increased DMT1 mRNA and function, we suspect that the lung may have evolved a specific response to iron to protect the epithelial surface from oxidative stress. Although iron is essential for almost every aspect of normal cell function, the same properties that allow it to catalyze numerous redox reactions also make it a threat to life via a generation of reactive oxygen species. Management of iron in particles is also critical to minimize the metal ions’ availability to microbial invaders that may arrive with the same particles (29). The lung minimizes these threats by sequestering metal that would otherwise be available for undesirable reactions. For instance, intracellular iron sequestration by ferritin limits its capacity to generate ROS and prevents cellular injury due to oxidative stress (17). This detoxification pathway requires that iron be transported across the plasma membrane. Usually, exposure to excess iron stimulates many cells to downregulate the expression of transferrin receptor (22) and, in the intestinal epithelium, to downregulate DMT1, restricting iron uptake in either case. Here, however, we demonstrate that control of DMT1 expression in respiratory epithelial cells differs from that in the intestine because –IRE mRNA and protein are upregulated by iron, resulting in cellular iron uptake and limiting the ROS generated by iron and other redox-active metals.

Vanadium compounds compete with iron for uptake by respiratory epithelial cells (28). In our current study, DMT1 downregulation after exposing BEAS-2B or normal rat lungs to VOSO₄ suggests that this metal interferes with normal DMT1 expression. Therefore, after exposure to vanadium, DMT1
expression and metal uptake are persistently decreased. Down-regulation of DMT1 after incubation of BEAS-2B cells with vanadium suggests toxicity by this metal that cannot be readily contained by the cells’ resources. Alternatively, vanadium uptake could free internal iron that is bound to intracellular ligands and increase the labile iron pool. This scenario would also make it beneficial to decrease vanadium uptake. The mechanisms of this effect of vanadium on DMT1 expression are not yet known, but vanadium does have well-known effects on cell signaling, such as inhibition of signaling cascade phosphatases (24). Regardless of mechanism and rationale, there is a clear correlation between DMT1 and vanadium uptake. We hypothesize that DMT1 transports vanadium and vanadyl is likely the form taken up.

In contrast to the +IRE DMT1, for which expression might be controlled at the posttranscriptional level (30), our findings implicate transcriptional control of −IRE DMT1 expression with changes in mRNA preceding alterations in protein expression. Such regulation of protein usually occurs at initiation of transcription, and the control elements affect the interaction of RNA polymerase with the gene promoter. The 5′-regulatory region of human DMT1 contains five potential metal response elements (MRE), three potential Sp1-binding sites, two potential hypoxia-inducible factor-1 binding sites, and one interferon regulatory element (12). Comparable to metallothionein, which contains numerous MRE binding sites in the promoter region and can be regulated by iron (3, 14), the presence of multiple MRE sites in DMT1 could confer rapid induction of message to iron exposure. On the other hand, metallothionein mRNA expression is not increased by vanadium, and this metal may not affect activation of MRE directly (1). Sp transcription factors are also involved in the expression of some iron-related proteins (19, 25). The discovery of an alternative exon 1A (10) upstream of the exon 1B promoter identified by Lee et al. (12) implies that there is also another promoter 5′ to exon 1A. This promoter could also have important relevant regulatory elements.

Immunohistochemistry demonstrated increased −IRE DMT1 expression by all epithelial cells after instillation of FAC into the lungs of animals. VOSO₄ had the opposite effect and diminished expression of −IRE DMT1 in the lung after exposure. The dose of VOSO₄ that can be employed in living animals is small due to lung damage. Metal uptake and oxidative stress after exposure to the particle correlated with expression of −IRE DMT1 in BEAS-2B cells. After the introduction of oil fly ash in vivo, metal transport out of the lower respiratory tract associated with the particle tracked that of the in vitro studies using FAC and VOSO₄, in which the preexposures respectively increased and decreased metal clearance. In vivo, lung injury after the particle was inversely related to metal transport, and lung damage decreased after induction of −IRE DMT1. The more rapid uptake of the metal associated with −IRE DMT1 appears to reflect more efficient control of oxidative stress and lung injury related to ROS production catalyzed by metals in the particle. Previous studies suggest that increased expression of −IRE DMT1 both increases iron uptake by resident cells of the lower respiratory tract and elevates expression of the storage protein ferritin in the same cells (6, 31).

Exposing lower respiratory tract cells to vanadium diminishes −IRE DMT1 mRNA and protein expression plus iron transport capacity. This effect may contribute to the recognized
toxicity of vanadium in the lung through downregulation of pathways involving not only its own transport but also other metals (and the control of untoward biological effects). Clearance of these metals by the lung is thus compromised by exposure to oil fly ash or other vanadium-containing compounds. In addition, the inability of vanadium-exposed lung cells to keep iron away from microbes portends uncontrolled microbial growth (15), and vanadium exposure is indeed associated with lung infections (20). On the other hand, pre-exposure of the lung to iron could result in tolerance to environmental challenges (including particles) with upregulation of -IRE DMT1 and mitigation of subsequent lung injury through better capacity to transport metal and control oxidative stress. This induction may explain an observed protective effect of air pollution particles in certain lung injuries (27). Our data on the responses of the Belgrade rat demonstrate that transport activity of DMT1 is critical for protection.

Table 1. Cell lipid peroxide (acetaldehyde) generation (nmol/20 μl cell lysate) after oil fly ash

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<thead>
<tr>
<th>Pretreatment</th>
<th>Exposure to Medium</th>
<th>Exposure to Ash</th>
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<tbody>
<tr>
<td>Medium</td>
<td>1.73±0.45</td>
<td>3.51±0.13</td>
</tr>
<tr>
<td>Iron</td>
<td>2.18±0.62</td>
<td>2.46±0.25*</td>
</tr>
<tr>
<td>Vanadium</td>
<td>2.60±0.38</td>
<td>4.97±0.43*</td>
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Values are means ± SE. BEAS-2B cells were preexposed to medium, 100 μM ferric ammonium citrate (FAC), or 50 μM vanadyl sulfate (VOSO₄) for 4 h to induce changes and then incubated with medium for 20 h and next exposed to either medium or 100 μg/ml oil fly ash in HBSS for 4 h to test for peroxide production (n = 2/group). After being washed with HBSS, the cells were scraped into 2,4-dinitrophenylhydrazine solution, and aldehydes were quantified by the HPLC system. Pretreatment of BEAS-2B cells with FAC diminished acetaldehyde generation after exposure to oil fly ash, whereas VOSO₄ had the opposite effect. *Significant difference compared with cells pretreated with medium and exposed to ash; n = 4/group.

Fig. 5. Metal uptake by BEAS-2B cells from either defined medium or medium containing oil fly ash. Iron (A and C) and vanadium (B and D) uptake were measured after cells were preexposed to either medium, 100 μM FAC, or 50 μM VOSO₄ for 4 h to induce responses and then incubated in medium. Twenty hours later, cells were incubated with either 100 μM FAC (A), 50 μM VOSO₄ (B), or 100 μg/ml oil fly ash (C and D). The concentrations of iron and vanadium in the medium were measured to determine how much metal had been transported intracellularly. *Significant decrease relative to preexposure to medium; n = 6 per treatment per time point.
GRANTS
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