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# The influence of high iron diet on rat lung manganese absorption

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**Abstract**

Individuals chronically exposed to manganese are at high risk for neurotoxic effects of this metal. A primary route of exposure is through respiration, although little is known about pulmonary uptake of metals or factors that modify this process. High dietary iron levels inversely affect intestinal uptake of manganese, and a major goal of this study was to determine if dietary iron loading could increase lung non-heme iron levels and alter manganese absorption. Rats were fed a high iron (1% carbonyl iron) or control diet for 4 weeks. Lung non-heme iron levels increased ~2-fold in rats fed the high iron diet. To determine if iron-loading affected manganese uptake, $^{54}$Mn was administered by intratracheal (it) instillation or intravenous (iv) injection for pharmacokinetic studies. $^{54}$Mn absorption from the lungs to the blood was lower in it-instilled rats fed the 1% carbonyl iron diet. Pharmacokinetics of iv-injected $^{54}$Mn revealed that the isotope was cleared more rapidly from the blood of iron-loaded rats. In situ analysis of divalent metal transporter-1 (DMT1) expression in lung detected mRNA in airway epithelium and bronchus-associated lymphatic tissue (BALT). Staining of the latter was significantly reduced in rats fed the high iron diet. In situ analysis of transferrin receptor (TfR) mRNA showed staining in BALT alone. These data demonstrate that manganese absorption from the lungs to the blood can be modified by iron status and the route of administration.


*Keywords:* Manganese; Iron; Metal pharmacokinetics; DMT1; Transferrin receptor; Lung metal transport

## Introduction

Chronic manganese exposure produces extra-pyramidal dysfunction (manganism) that resembles the dystonic movement associated with Parkinson's disease. Occupational exposures to high manganese-containing dusts or aerosols place workers at a higher risk for manganism (Pal et al., 1999) and it has been speculated that manganese toxicity contributes to the development of Parkinson's disease (Gorell et al., 1999; Powers et al., 2003). To better understand the origins of manganese neurotoxicity, it is necessary to elucidate the molecular mechanisms responsible for the metal's absorption. Deposition of manganese in the brain is dependent on the route of exposure and Roels et al. (1997) have determined that intratracheal instillation results in significantly higher brain manganese levels compared to ingestion. However, while the molecular details of intestinal manganese absorption have begun to emerge, little is known about the mechanism(s) responsible for uptake from the respiratory tract.

It has been established that an inverse relationship exists between body iron stores and manganese absorption from the gut (Diez-Ewald et al., 1968; Finley, 1999). For example, diets high in iron have been shown to suppress manganese absorption while low-iron diets increase manganese uptake (Pollack et al., 1965; Diez-Ewald et al., 1968; Gruden, 1986) including that to the brain (Shukla et al., 1989; Erikson et al., 2002). The metabolic interactions between iron and manganese are thought to arise from transport pathways shared by the two metals. Manganese and iron uptake appear to involve divalent metal transporter-

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1 (DMT1) (Chua and Morgan, 1997) as well as transferrin (Tf) and transferrin receptors (TfR) (Suarez and Eriksson, 1993; Malecki et al., 1999a, 1999b; Roth et al., 2002). Levels of these factors reflect body iron status due to iron-responsive regulation and become down-regulated under high iron conditions to reduce net iron assimilation (Eisenstein, 2000). Since it has been argued that the molecular elements responsible for intestinal iron and manganese absorption might play similar roles in the lung (Turi et al., 2004), we undertook this study to determine the effects of dietary iron loading on lung non-heme iron content, levels of DMT1 and TfR expression, and pulmonary manganese absorption.

## Methods

*Animals and diets.* All animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Male Sprague–Dawley rats (3-week-old) were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and housed in microisolator cages with free access to water and rodent chow. The animals were randomly divided into a 4-week iron-loading group and age-matched controls. Control rats were fed a diet containing 210 ppm iron (Purina Test diet #5053, PharmaServ, Framingham, MA). To induce iron loading, rats were fed the same diet modified to contain 1% carbonyl iron (10,000 ppm iron).

*Pharmacokinetics and tissue uptake of $^{54}Mn$.* Rats were anesthetized with vaporized halothane (Halocarbons Lab, North Augusta, SC) prior to delivery of radioisotope. $^{54}MnCl_2$ was obtained from Perkin-Elmer/NEN (Boston, MA) and diluted in phosphate-buffered saline (PBS) for intratracheal (it) instillation (10 μCi/ml) as described by (Brain et al., 1976). The final delivery volume was 1.5 ml/kg body weight. For intravenous (iv) injection into the penile vein, $^{54}MnCl_2$ was diluted in PBS to 30 μCi/ml and a volume of 0.5 ml/kg body weight was administered. To determine the pharmacokinetics of blood manganese levels after it and iv administration, samples were collected from the tail artery 5 min to 4 h post-instillation or injection. Rats were humanely killed 4 h after administration of isotope and tissue samples of lung, liver, spleen, kidney and brain were collected for the measurement of nonheme iron and the amount of associated $^{54}Mn$. All tissue and blood samples were weighed, radioactivity was measured in a gamma counter (Cobra Quantum, Packard Instruments, IL) and uptake was calculated as a percentage of the instilled or injected dose of $^{54}Mn$. For blood, skeletal muscle and bone marrow, these calculations were based on estimations of tissue weight as a fraction of body weight of 7%, 45% and 3%, respectively (Diluzio and Zilversmit, 1955). Non-heme iron was determined using the method of Torrance and Bothwell (1968) and calculated as μg iron per gram of wet tissue weight.

Table 1
Characteristics of male rats fed control and high iron (1% carbonyl) diets

| | Age (weeks) | Hematocrit (%) | Body Weight (g) |
|---|---|---|---|
| Control | 7 | 43.96 ± 1.081 | 356.40 ± 23.63 |
| 1% Carbonyl Iron | 7 | 45.70 ± 0.787 | 337.45 ± 11.66 |

Values are means ± SEM ($n$ = 5).

*In situ hybridization.* After 4 weeks on the high iron diet as described above, rats were humanely killed and tracheas were cannulated with a blunt 18-gauge needle and syringe filled with O.C.T. compound (Sakura Finetek USA, Torrence, CA) pre-warmed to 37 °C. Lungs were filled with O.C.T., cut laterally into sections, mounted in O.C.T., snap-frozen in 2-methylbutane, chilled on dry ice, and stored at −80 °C. Ten μm-thick sections were cut on a cryotome and stored at −20 °C until processed. In situ hybridization was performed as described elsewhere (Gunshin et al., 1997). Briefly, digoxigenin-labeled sense and antisense cRNA probes were transcribed from plasmid pT7T3D-Pac-rat DMT1 (GenBank accession no. AA819678, American Type Culture Collection, Manassas, VA) and plasmid pT7T3D-Pac-rat transferrin receptor (TfR) (GenBank accession no. AI454017, Resgen, Huntsville, AL). Transcripts were shortened to an average length of 200–400 bp by alkali hydrolysis. Sections were incubated with sense or antisense probe (~200 ng/ml) in hybridization buffer (50% formamide, 5× SSC, 2% blocking reagent, 0.02% SDS, 0.1% N-laurylsarcosine). Hybridized probes were detected using anti-digoxigenin Fab fragments and BCIP/NBT substrate. Sections were incubated in substrate solution for 42 h, then rinsed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and mounted in 50% PBS/glycerol.

*Statistical analysis.* Data shown are the means ± SEM. Pharmacokinetic results and tissue distribution of instilled and injected isotope were evaluated by multivariate analysis of variance (MANOVA) using the General Linear Model Procedure (SAS statistical analysis software, SAS Institute, Cary, NC). $P$ values for tissue $^{54}Mn$ absorption were determined by F test using all organ values as responses. Comparisons of hematocrits, body weights and tissue non-heme iron levels between control and iron-loaded rats (Tables 1 and 2) were evaluated by Student's unpaired $t$ test. Statistical significance between mean values was taken as $P$ less than 0.05.

## Results

### *Body weights, hematocrit and nonheme iron levels*

Characteristics of male Sprague–Dawley rats fed a high iron diet (1% carbonyl iron) for 4 weeks are shown in Table 1. Body weights and hematocrit values were not significantly different from controls. Non-heme iron levels in the lungs were increased ~2-fold in rats fed the high iron diet (Table 2).

Table 2
Nonheme iron levels in tissues from rats fed control and high iron diet (1% carbonyl)

| | Lung | Liver | Kidney | Spleen | Brain |
|---|---|---|---|---|---|
| Control | 15.6 ± 4.06 | 80.00 ± 4.62 | 58.8 ± 15.917 | 66.7 ± 12.85 | 13.8 ± 3.14 |
| 1% carbonyl iron | 40.80 ± 1.277* | 278.9 ± 53.58* | 70.6 ± 0.866 | 366.9 ± 67.55* | 19.2 ± 0.75 |

Values (μg iron/gram wet tissue) are means ± SEM ($n$ = 5).
* $P < 0.05$.

Consistent with previous reports (Chua and Morgan, 1996; Vayenas et al., 1998), non-heme iron levels were also significantly increased in the liver and spleen. Although a trend towards higher non-heme iron levels was noted for brain tissue, this effect was not statistically significant.

### *Pharmacokinetics of intratracheally-instilled $^{54}$Mn*

To compare the pharmacokinetics of manganese absorption from the lungs to the blood in iron-loaded and control rats, $^{54}$Mn was it-instilled and isotope levels were determined in blood samples drawn 5 min to 4 h post-instillation. Compared to age-matched controls, rats fed the high iron diet had significantly lower levels of blood $^{54}$Mn throughout the entire time course (Fig. 1). The effect of diet was statistically significant (MANOVA, $P < 0.01$). There was also a significant difference in the amount of $^{54}$Mn remaining in the blood at 4 h in that rats fed the 1% carbonyl diet had lower levels of circulating isotope (0.033% versus 0.093% instilled dose, $P < 0.05$).

### *Pharmacokinetics of intravenously-injected $^{54}$Mn*

In order to evaluate potential contributions due to the clearance of $^{54}$Mn from the blood to tissues to the pharmacokinetics of pulmonary metal absorption, blood isotope levels were determined after iv injection. In rats fed the high iron diet (Fig. 2), iv-injected $^{54}$Mn disappeared more rapidly compared to the control groups and the effect of diet was statistically significant (MANOVA; $P < 0.05$). These results are consistent with previous reports that indicate tissue uptake of manganese is enhanced by iron-loading (Chua and Morgan, 1996; Vayenas et al., 1998).

### *Tissue absorption of intratracheally-instilled versus intravenously-injected $^{54}$Mn*

Four hours after administration of $^{54}$Mn, rats were humanely killed and tissues were collected to determine the amount of isotope taken up by the organs listed in Table 3. A significant amount of instilled $^{54}$Mn remained in the lungs of rats that were fed the 1% carbonyl iron diet (79.181% compared to 57.940% instilled dose in age-matched control animals, $P < 0.05$). This observation is consistent with reduced levels of isotope circulating in blood at 4 h (Fig. 1). Due to reduced uptake from the lungs, tissue $^{54}$Mn levels were generally lower compared to age-matched controls, although only values for the small intestine reached statistical significance. One exception was that bone marrow had significantly higher levels of $^{54}$Mn in rats fed the high iron diet. In contrast, tissue levels of iv-injected isotope tended to be higher in rats fed the high iron diet (Table 3), compatible with enhanced tissue uptake due to iron-loading that has been shown by others (Chua and Morgan, 1996; Vayenas et al., 1998), and the more rapid clearance of isotope observed after iv injection (Fig. 2).

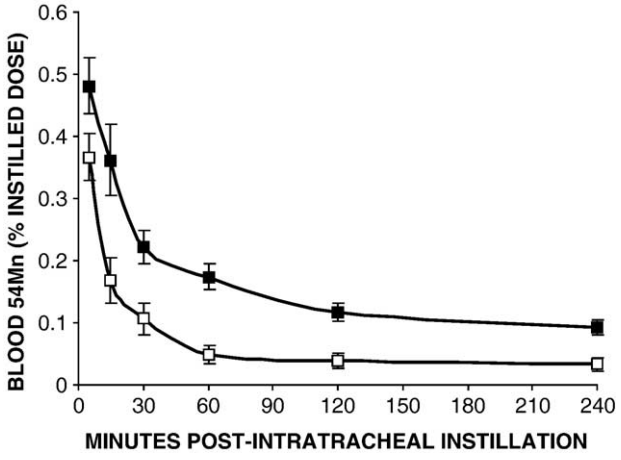


Fig. 1. Vascular kinetics of it-instilled $^{54}$Mn in rats fed control or high iron diet. Isotope was administered intratracheally to rats fed control diet (closed symbol; $n$ = 13) or diet supplemented with 1% carbonyl iron (open symbol; $n$ = 9). Blood samples were drawn at the indicated times and blood $^{54}$Mn levels are determined as % instilled dose. Each data point is the mean ± SEM.

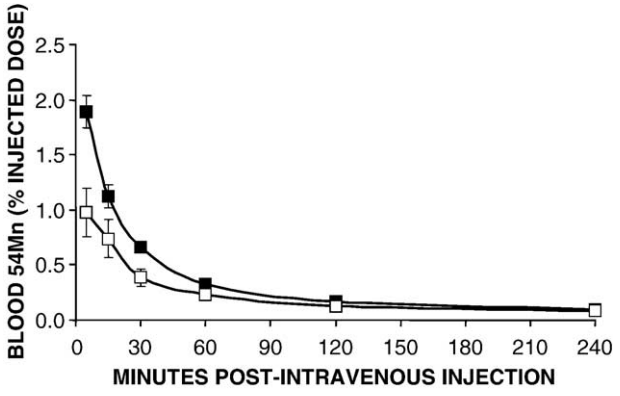


Fig. 2. Vascular kinetics of iv-injected $^{54}$Mn in rats fed control or high diet. Isotope was injected into the penile vein of rats fed control diet (closed symbol) or diet supplemented with 1% carbonyl iron (open symbol). Blood samples were withdrawn from the lateral tail artery at the indicated times and blood $^{54}$Mn levels are determined as % instilled dose. Each data point is the mean ± SEM for five rats.

Table 3
Tissue distribution of $^{54}$Mn 4 h after instillation or intravenous injection of rats fed control or high iron (1% carbonyl) diet

| | Intratracheal | | Intravenous | |
|---|---|---|---|---|
| | Control diet[a] | High iron diet[b] (1% carbonyl iron) | Control diet | High iron diet (1% carbonyl iron) |
| Brain | 0.046 ± 0.006 | 0.030 ± 0.002 | 0.211 ± 0.011 | 0.231 ± 0.016 |
| Liver | 4.898 ± 0.897 | 5.342 ± 0.338 | 22.414 ± 1.360 | 22.399 ± 1.256 |
| Spleen | 0.189 ± 0.028 | 0.142 ± 0.005 | 0.612 ± 0.040 | 0.712 ± 0.077 |
| Lungs | 57.940 ± 2.488 | 79.181 ± 0.275* | 0.921 ± 0.033 | 1.138 ± 0.062* |
| Kidney | 2.560 ± 0.314 | 1.885 ± 0.049 | 8.001 ± 0.487 | 9.982 ± 0.467* |
| Heart | 0.314 ± 0.049 | 0.245 ± 0.009 | 1.247 ± 0.036 | 1.319 ± 0.073 |
| Bone Marrow | 0.776 ± 0.085 | 1.287 ± 0.098* | 2.855 ± 0.164 | 3.149 ± 0.154 |
| Skeletal Muscle | 4.291 ± 0.720 | 4.732 ± 0.346 | 13.334 ± 0.793 | 12.526 ± 0.979 |
| Stomach | 1.233 ± 0.386 | 0.383 ± 0.011 | 1.714 ± 0.114 | 1.500 ± 0.069 |
| Small Intestine | 6.443 ± 0.826 | 2.644 ± 0.444* | 16.125 ± 0.694 | 15.887 ± 1.399 |
| Large Intestine | 2.865 ± 0.741 | 2.672 ± 0.262 | 6.638 ± 0.572 | 6.423 ± 0.717 |

Values (% instilled dose) are means ± SEM ($n = 5$ except [a]$n = 6–8$ and [b]$n = 4$; MANOVA, *$P < 0.05$). [a]Results for tissue distribution after instillation of control rats are combined with control data from another study (Brain et al., submitted for publication).

*DMT1 and TfR expression in the rat lung*

Expression levels and distribution of DMT1 and TfR mRNA were evaluated by in situ hybridization of lungs from rats fed control or 1% carbonyl iron diet. DMT1 mRNA was detected in airway epithelium, bronchus-associated lymphoid tissue (BALT) and macrophages (Fig. 3). Compared to controls, rats fed the high iron diet had significantly less staining in BALT while staining of pulmonary epithelium was similar between the two groups. In contrast to DMT1 staining, the only region of the lung that stained positively for TfR mRNA was BALT but the

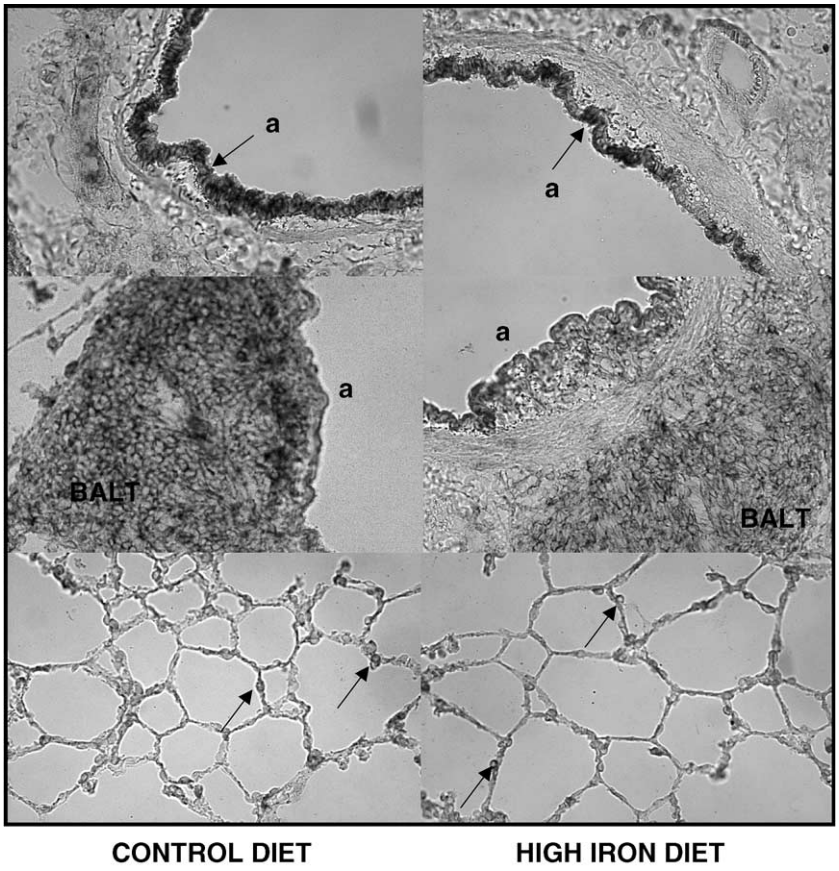


Fig. 3. In situ hybridization for lung DMT1 mRNA levels in rats fed control or high iron diet. Message levels for DMT1 were examined in the lungs for rats fed control diet (left panels) or 1% carbonyl diet (right panels). Bronchial airways (a), bronchus-associated lymphoid tissue (BALT) and macrophages (arrows) are labeled. Magnification is 25×.

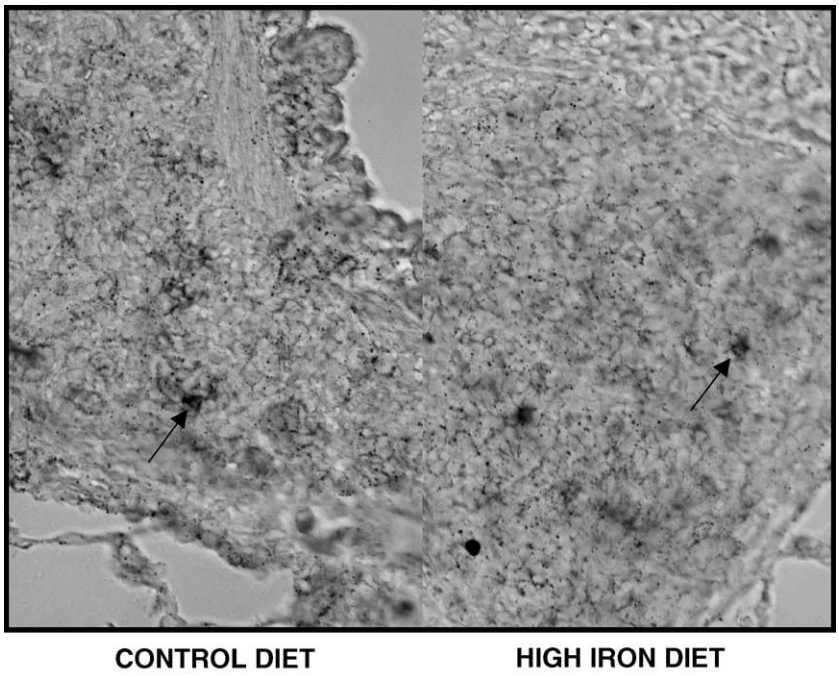


Fig. 4. In situ hybridization for lung TfR mRNA levels. Message levels for TfR were examined in the lungs of rats fed control diet or high iron (% carbonyl) diet as indicated. Magnification is 25×. Low levels of staining that were observed were only detected in BALT.

relatively low levels that were detected did not change significantly with diet (Fig. 4).

## Discussion

The metabolic relationships between manganese and iron are well established (Roth and Garrick, 2003). These essential trace elements have similar valency states under physiological conditions (II and III) and atomic radii that are close in size (0.80 and 0.74 Å). The relationship between the physiochemical properties of the two metals most likely contributes to the fact that they share transport mechanisms. It has been shown that DMT1 mediates cellular import of both iron and manganese (Gunshin et al., 1997; Forbes and Gros, 2003) and that anti-DMT1 antisera block uptake of both metals in the divalent state (Conrad et al., 2000). Dietary absorption of both iron and manganese is impaired in the Belgrade rat (Chua and Morgan, 1997), an animal model of anemia that harbors a point mutation in DMT1 leading to altered activity and expression levels (Fleming et al., 1998; Su et al., 1998). Competitive uptake of Fe(II) and Mn(II) by DMT1 helps to explain decreases in intestinal manganese absorption observed with high iron diets (Davis et al., 1992a, 1992b) as well as the inhibition of dietary iron absorption by high manganese (Rossander-Hulten et al., 1991). The fact that DMT1 levels are influenced by iron content of the diet (Oates et al., 2000; Yeh et al., 2000; Frazer et al., 2003) also may contribute to the competitive effect of iron on manganese absorption. Once absorbed from the diet, both Fe(III) and Mn(III) can bind Tf to be delivered to cells via interactions with TfRs (Suarez and Eriksson, 1993; Malecki et al., 1999a; Roth et al., 2002). After endocytosis of the Tf–TfR complex, DMT1 is also thought to be involved in the uptake of Tf-bound iron (Fleming et al., 1998; Su et al., 1998; Canonne-Hergaux et al., 2001).

Levels of DMT1 and TfR mRNA are regulated in response to iron status (Eisenstein, 2000). Up-regulated expression of DMT1 due to iron deficiency (Gunshin et al., 1997; Canonne-Hergaux et al., 1999) provides an explanation for enhanced uptake of manganese in anemic animals (Thomson et al., 1971). It has been proposed that elements of the intestinal transport pathway also play a role in airway iron absorption (Turi et al., 2004). Since mRNA levels of DMT1 and TfR are known to be directly influenced by dietary iron levels (Eisenstein, 2000) and each of these transporters have been implicated in manganese uptake (Roth and Garrick, 2003), our studies sought to determine if experimental iron overload induced by diet could modify the absorption of instilled manganese and expression of these transporters by the lung. To this end, rats fed a diet containing 1% carbonyl iron for 4 weeks had increased non-heme iron levels in the lungs (~2-fold). To our knowledge, this is the first report of increased lung tissue iron levels in response to dietary overload; Yokoi et al. (1991) have previously reported that dietary iron deficiency reduced lung iron levels.

The results of the pharmacokinetic experiments reported here demonstrate that rats fed the high iron diet had decreased absorption of manganese from the lungs to the blood compared to age-matched controls. In contrast, iv-

injected $^{54}$Mn appeared to be more rapidly cleared from circulation in rats fed the high diet and this was accompanied by a general increase in tissue isotope levels in agreement with previous studies (Chua and Morgan, 1996; Vayenas et al., 1998). The diminished transport of manganese across the air–blood barrier indicates that this metal is taken up by an iron-responsive mechanism and clearly demonstrates that the pathway of pulmonary manganese absorption can be down-regulated under iron-loading conditions.

To identify potential changes in DMT1 expression in the lung in response to the high iron diet, we used in situ hybridization to assess transcript levels. DMT1 was found to be expressed in airway epithelium, BALT and alveolar macrophages. In rats fed the high iron diet, DMT1 levels were significantly reduced in BALT, implicating this tissue as an iron-responsive region of the lung that could potentially be involved in metal uptake. Consistent with this idea, TfR mRNA was only detected in BALT; although no detectable change in expression was observed in rats fed the high iron diet, expression levels were quite low. Further work is clearly necessary to define the possible role of DMT1, as well as Tf/TfR, in lung metal absorption. Recent studies have indicated that DMT1 is unlikely to be involved in manganese uptake across the blood–brain barrier (Crossgrove and Yokel, 2004; Moos and Morgan, 2004); our observations are compatible with these data in that $^{54}$Mn uptake into the brain after it and iv instillation was not significantly different in iron-loaded rats.

Ghio and coworkers (Ghio et al., 2000; Wang et al., 2002; Yang et al., 2002) have shown enhanced expression of DMT1 in response to instilled residual oil fly ash (ROFA) and other metal-containing particles, leading to the postulation that levels of transport factors are increased to promote the clearance of iron from the airways to avoid oxidative damage (Turi et al., 2004). Our study contrasts with this model since it demonstrates that DMT1 levels and metal uptake from the lung can be influenced by systemic iron-loading in an inverse manner. More recently, however, Wang et al. (in press) have reported that DMT1 expression in bronchial epithelial cells is increased by inflammatory cytokines (tumor necrosis factor-α and interferon-γ) and lipopolysaccharide. Thus, it is possible that the enhanced expression of DMT1 observed in their earlier studies was, at least in part, due to an inflammatory response. Unfortunately, these investigators did not measure non-heme iron levels so it is not clear that DMT1 expression was regulated by a change in intracellular iron status. Our study shows that decreased $^{54}$Mn uptake from the lungs to the blood is associated with increased tissue iron-loading. Evidence from our study further suggests that a synergistic relationship exists between iron and manganese transport systems in the lungs. Because of these effects, the neurotoxicity of occupational exposures to airborne manganese in environments such as ore processing, metallurgy, battery manufacture and agriculture may be altered by body iron status. These are important parameters to consider when assessing the impact of manganese exposure and its associated neurotoxicity.

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