Olfactory ferric and ferrous iron absorption in iron-deficient rats

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Ruvin Kumara VM, Wessling-Resnick M. Olfactory ferric and ferrous iron absorption in iron-deficient rats. Am J Physiol Lung Cell Mol Physiol 302: L1280–L1286, 2012.—The absorption of metals from the nasal cavity to the blood involves a DMT1 (divalent metal transporter-1)-mediated transport pathway. Despite the established olfactory metal uptake pathway and the characterized role for DMT1, it has been generally assumed that iron is not assimilated from the nasal cavity to the brain. Rao et al. (22) have shown that aerosolized 59FeSO4 is rather poorly absorbed and that the radioisotope associates with transferrin in the mucosa. However, there are several reasons to reexamine olfactory uptake of iron. Like manganese, iron is also present in welding fume, being the metal in the largest proportion in mild steel welding fume, for example (10). Although manganese toxicity is well established, not all adverse health effects attributable to welding exposure are associated with this metal, suggesting that other components in welding fume may cause damage. In one study, the loss of smell in welders was associated with time spent welding, but workers with higher levels blood manganese had better olfactory function (2). Another consideration is the speciation of iron: gas metal arc welding fume contains 79% iron (III) oxide and 31% iron (II) oxide. Studies by Wang et al. (34) suggest that ferric oxide particles can be transported by the olfactory pathway to the central nervous system and cause oxidative damage. Finally, our findings on iron-responsive regulation of DMT1 suggest that anemia could modify its function to enhance metal uptake in exposed workers with nutritional iron deficiency (30).


Iron is an essential nutrient required for many physiological processes. Nutritional iron deficiency is the most common cause of anemia, which affects more than 1.5 billion people globally (36). However, iron is also a toxic metal associated with Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and amyotrophic lateral sclerosis, among other disorders (20, 23, 24, 26). Iron deficiency enhances dietary uptake to resolve the anemic state, but other routes of transport including uptake from the lungs are also highly regulated by iron status (4, 13, 28). This paradox raises questions about how iron deficiency may modify the potential toxicity associated with metal uptake to the brain across the olfactory pathway.

Inhalation is an important route of occupational exposure and metal toxicity. Welders, for example, are exposed to fumes composed of iron, chromium, manganese, aluminum, nickel, and cadmium (1). Much epidemiological research has focused on olfactory manganese uptake and its toxicity, which is associated with tremors in a Parkinson’s-like disease called manganese found in welders and other workers (3, 25). Absorption of manganese and other metals via olfactory tissues to the brain is well documented (7, 21, 30–32). In the case of manganese, we have shown that metal absorption is upregulated by iron deficiency and that the major intestinal iron transporter divalent metal transporter-1 (DMT1) plays a role (30). Although DMT1 functions in iron absorption, it interacts with a substrate profile ranked on currents as: Cd2+, Co2+, Fe2+, Mn2+ > Ni2+, V3+ > Pb2+ (17). Belgrade rats (b/b), which have defective iron uptake attributable to a point mutation in DMT1 (9), absorb less intranasally instilled 54Mn compared with iron-deficient heterozygous +/b counterparts, whereas the latter absorb more radioisotope compared with iron-sufficient +/+ control rats (30). Iron-deficient rats have increased levels of DMT1 localized to both the lumen microvilli and end feet of sustentacular cells of the olfactory epithelium (30). Sustentacular cells are necessary for manganese transport to the blood, and intact axonal projections are regulated by manganese uptake. The olfactory bulb and brain (31). Thus DMT1 would appear to be a critical mediator of metal exposures via the olfactory pathway.

Despite the established olfactory metal uptake pathway and the characterized role for DMT1, it has been generally assumed that iron is not assimilated from the nasal cavity to the brain. Rao et al. (22) have shown that aerosolized 59FeSO4 is rather poorly absorbed and that the radioisotope associates with transferrin in the mucosa. However, there are several reasons to reexamine olfactory uptake of iron. Like manganese, iron is also present in welding fume, being the metal in the largest proportion in mild steel welding fume, for example (10).

Although manganese toxicity is well established, not all adverse health effects attributable to welding exposure are associated with this metal, suggesting that other components in welding fume may cause damage. In one study, the loss of smell in welders was associated with time spent welding, but workers with higher levels blood manganese had better olfactory function (2). Another consideration is the speciation of iron: gas metal arc welding fume contains 79% iron (III) oxide and 31% iron (II) oxide. Studies by Wang et al. (34) suggest that ferric oxide particles can be transported by the olfactory pathway to the central nervous system and cause oxidative damage. Finally, our findings on iron-responsive regulation of DMT1 suggest that anemia could modify its function to enhance metal uptake in exposed workers with nutritional iron deficiency (30).
MATERIALS AND METHODS

Animals and diets. Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Three-week-old Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed with a 12-h:12-h light/dark cycle. To induce dietary iron deficiency, rats were fed chow containing 4–7 ppm iron (TD 99397; Harlan Teklad, Madison, WI) for 3 wk. Food consumption was measured, and control rats were fed ~125% of this amount using a control diet containing 220 ppm iron (PicoLab5053; LabDiet, Richmond, IN). Belgrade rats were fed on food and water ad libitum and maintained on a 12-h:12-h light/dark cycle. Mating pairs of female heterozygous (+/b) and male homozygous Belgrade (b/b) rats were fed an iron-supplemented diet containing 480–600 mg iron/kg (TD02385, Harlan Teklad). Female +/b rats were fed an iron-supplemented diet throughout pregnancy. At postnatal day 6, litters were cross-fostered to F344 Fischer dams (+/+; Charles River, Boston, MA) fed the control diet (PicoLab 5053; PharmaServ, Framingham, MA). Belgrade rats were phenotyped by their pallor at birth; hematocrit and tissue nonheme iron concentrations were measured as previously described (4). At the time of weaning, rats were divided into three groups; both b/b and control +/+ rats were fed iron-supplemented diet, and a separate cohort of +/+ rats were fed the iron-deficient diet.

Pharmacokinetic experiments. $^{59}$FeCl$_3$ was purchased from Perkin Elmer/New England Nuclear (Boston, MA) and diluted in 20 mM HEPES/Tris buffer containing nitrilotriacetic acid (NTA, 1:4 molar ratio). The pH was adjusted to 6.5 immediately before intranasal instillation for ferric iron uptake experiments. A similar $^{59}$Fe solution was prepared for ferrous iron uptake experiments except that ascorbic acid (1:50 molar ratio) was added to reduce ferric to ferrous iron. $^{59}$Fe was then administered to anesthetized rats by intranasal instillation (30 $\mu$Ci/kg body wt; 35 $\mu$g/kg), and blood samples were collected from the tail vein at 5, 15, 30, 60, 120, and 240 min postinstillation. Homozygous Belgrade rats, iron-deficient heterozygous rats, and control heterozygous rats were humanely killed 4 h postinstillation and exsanguinated. Brain, liver, heart, lungs, esophagus, trachea, sternum, spleen, muscle, kidneys, stomach, stomach content, duodenum, jejunum, ileum, cecum, and large intestine were collected. All tissues and blood samples were weighed, and radioactivity was measured in a Packard $^{1281}$counter (Perkin Elmer Wallac Wizard 1470–020). $^{59}$Fe level was calculated as a percentage of the instilled dose based on tissue weight to body weight. In the case of blood, bone marrow (sternum), and skeletal muscle, estimation of 7%, 3%, and 45% was used, respectively.

Western blot analysis. Snap-frozen olfactory bulbs from iron-deficient and control rats were homogenized in 50 mM mannitol, 2 mM HEPES, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.2, using a Tissue Tearor (Biospec Products, Bartlesville, OK). The homogenate was centrifuged at 1,500 g for 5 min, and the supernatant was centrifuged for 1 h at 15,000 g to obtain the crude membrane fraction for detergent extraction using 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.5 mM PMSF. Protein (50 $\mu$g) was electro-

Fig. 1. Vascular kinetics of intranasally instilled radioactive ferrous iron in iron-deficient and control rats. Hematocrit (A), liver nonheme iron (B), brain nonheme iron (C), and body weight (D) are shown (top) for iron-deficient (solid bars) and control (open bars) rats. Vascular kinetics of radioactive iron was measured after intranasal instillation of 30 $\mu$Ci/kg body wt $^{59}$Fe reduced to the ferrous form by a 50-fold molar excess of ascorbate immediately before administration. Bottom: blood samples were collected from the tail vein at 5, 15, 30, 60, 120, and 240 min of iron-deficient (▲) and control (■) rats to determine the percentage of dose present in blood. Results shown are the means ± SE (n = 6). **$P < 0.001$, *$P < 0.05$, ANOVA.
phoresed on a 4–15% gradient SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were incubated with anti-DMT1 (1:1,000, a kind gift of Dr. Phillippe Gros, McGill University), washed, and developed using anti-rabbit horseradish peroxidase-linked antibody (1:6,000 dilution; Amersham, Buckinghamshire, UK) using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL) and visualized on film. As a loading control, blots were reprobed with anti-actin (1:20,000 dilution; MP Biomedical, Solon, OH). ImageJ (version 1.44) software was used to determine film intensity, and protein levels were normalized to actin. Statistical analysis. Data are expressed as means ± SE. Data were analyzed by one-way ANOVA and Tukey’s or Dunnett’s tests where appropriate, using IBM SPSS version 19 statistical software; differences were considered significant at $P < 0.05$.

RESULTS

Rats fed a low-iron diet for 3 wk had reduced hematocrit and nonheme iron levels in liver and brain compared with iron-sufficient control rats pair fed normal chow (Fig. 1, A–C). Body weights were similar for both cohorts (Fig. 1D). To measure olfactory absorption of $^{59}$Fe$^{2+}$ (ferrous iron), diluted ferric $^{59}$FeCl$_3$ was reduced to the ferrous form by addition of a 50-fold molar excess of ascorbic acid immediately before administration by intranasal instillation. $^{59}$Fe absorption from the nasal cavity to the blood was significantly greater at 2 h and 4 h in anemic rats compared with controls (Fig. 1, bottom).

To examine absorption of $^{59}$Fe$^{3+}$ (ferric iron), radioisotope was diluted and stably complexed with NTA at a 1:4 ratio. Previous studies of nontransferrin-bound iron uptake have shown efficient cellular iron uptake when this form of ferric iron was employed (14). The $^{59}$Fe:NTA complex was administered intranasally to a second cohort of iron-deficient and control rats with the physiological characteristics shown in Fig. 2, A–D. These data confirm that the iron-deficient rats had reduced hematocrit and liver and brain nonheme iron levels. One hour after intranasal instillation, $^{59}$Fe in the blood was significantly greater and increased over time in iron-deficient rats compared with controls, with 18.32 ± 3.67% and 4.81 ± 3.71% of the total instilled dose measured in the blood at 4 h, respectively (Fig. 2, bottom).

Comparison of results presented in Fig. 1 and 2 demonstrates that $^{59}$Fe administered in the ferrous form to the nasal cavity appeared in the blood at levels comparable to the time course observed for ferric form in control rats (e.g., 2.5 ± 1.6% vs. 4.81 ± 3.71% of the total instilled dose at 4 h, respec-

![Fig. 2. Vascular kinetics of intranasally instilled radioactive ferric iron in iron-deficient and control rats. Hematocrit (A), liver nonheme iron (B), brain nonheme iron (C), and body weight (D) are shown (top) for iron-deficient (solid bars) and control (open bars) rats. Vascular kinetics of radioactive ferric iron was measured after intranasal instillation of 30 mCi/kg body wt $^{59}$Fe:nitrilotriacetic acid complexed as described in MATERIALS AND METHODS. Bottom: blood samples were collected from the tail vain at 5, 15, 30, 60, 120, and 240 min of iron-deficient (▲) and control (■) rats. Results shown are the means ± SE (n = 10 iron-deficient rats and n = 8 control rats). **P < 0.001, *P < 0.05, ANOVA.)](http://ajplung.physiology.org/doi/10.1152/ajplung.00004.2012)
DMT1 function (29). After intranasal instillation, uptake of to the iron-loading anemia phenotype associated with loss of $b/b$ lower iron concentrations in the liver and the brain, whereas with control rats had reduced body weights and hematocrit values compared with control littermates. Both cohorts of $b/b$ littermates had reduced body weights and hematocrit values compared with control heterozygous rats. Although the $59\text{Fe}$ in control was significantly lower in anemic $+/b$ rats, other intestinal regions (duodenum, ileum, jejunum, and large intestine) and stomach contents were not different between the three cohorts, suggesting that, after administration in the ferric form, iron was absorbed from the olfactory system and not cleared from the gut attributable to the rat’s regurgitation and swallowing of isotope. Notably, $59\text{Fe}$ in whole brain was significantly increased in iron-deficient $+/b$ rats compared with control $+/b$ and $b/b$ littermates. Although the $59\text{Fe}$ content in cecum was significantly lower in anemic $+/b$ rats, Western blot experiments were performed. Figure 5 shows that DMT1 protein was increased in olfactory tissue from iron-deficient rats compared with controls.

**DISCUSSION**

Accumulating evidence indicates that olfactory tissues can absorb manganese, cobalt, cadmium, nickel, and mercury (32) and that metal absorption is enhanced by iron deficiency (4, 6, 30). Pharmacokinetic studies of metal uptake by Belgrade rats and immunolocalization of DMT1 in the olfactory epithelial regions of the nasal concha support the model that DMT1 is involved in manganese absorption and is responsible for enhanced uptake under low-iron conditions (30). Less is known about iron uptake, and previous work suggested ferrous iron was poorly absorbed (22). In our study, uptake of $59\text{Fe}$ from the nasal cavity to the blood was observed whether the radioisotope was administered in the ferrous or ferric form to iron-sufficient rats, but the amount assimilated was lower than observed for iron-deficient rats. These observations indicate that olfactory iron absorption does occur and is dependent on body iron status. In this tracer study, only small amounts of $59\text{Fe}$ were instilled into rats. Ferric iron uptake could be greater with much higher occupational exposures and should be further explored. High iron content can be detected in exhaled breath of workers (14). In a pilot study, we instilled $59\text{Fe}$ at a much higher dose (150 $\mu\text{Ci/kg body wt}$), and greater levels in the blood were observed (data not shown). Thus it should not be presumed that iron uptake does not occur under iron sufficiency conditions, especially for the ferric form present in occupational settings like welding.
Another important finding is that iron-deficient rats display significantly greater absorption of both ferrous and ferric iron. This observation is concordant with the enhanced olfactory manganese absorption reflecting upregulation of DMT1 (30). Because iron deficiency is one of the most prevalent nutritional deficiencies worldwide, rather simple measures could reduce the potential risk of oxidative damage to workers exposed to iron by routine monitoring of their hematological status and recommending iron supplements if a nutritional deficiency is detected.

Surprisingly, transfer of ferric iron from the nasal cavity to the blood appeared to be more effective than absorption of ferrous iron, raising questions about the role for DMT1 in this pathway because its transport substrate preference is the divalent form (12). Using the Belgrade rat model of DMT1 deficiency, our study showed that uptake of ferric iron by iron-deficient rats was dependent on DMT1 function because b/b rats failed to show significant absorption of radioisotope from the nasal cavity to the blood and, importantly, the brain. Western blot analysis of olfactory tissue confirmed that DMT1 was upregulated in iron-deficient rats compared with control rats. These combined results lead to the plausible model that DMT1 in olfactory tissues of iron-deficient rats enhances uptake of both ferric and ferrous iron (30, 31), which is then transferred to the olfactory bulb and brain or transported to blood capillaries. What factors promote ferric iron uptake by DMT1 remain to be identified, but in preliminary studies we have found iron-responsive expression of duodenal cytochrome B in olfactory tissue (data not shown). This ferrireductase reduces ferric to ferrous iron for DMT1-mediated uptake in the intestine (18). A different ferrireductase called Steap3 also functions with DMT1 to mediate assimilation of ferric iron from transferrin in the endolysosomal pathway (19). Ferric oxide particle dissolution can occur in the endolysosomal pathway, and it is possible that DMT1 functions here to release iron across the membrane where it can inflict oxidative damage to the cell. Rao et al. (22) administered ferrous iron by inhalation and found that most of the isotope remained in mucosa and was associated with transferrin, which binds iron (III). This suggests that ferrous and ferric iron may follow the same pathway involving the transferrin receptor. Transferrin receptors are also upregulated by iron deficiency and could enable its transfer across the olfactory epithelium. Subsequent mobilization of transferrin-bound iron would require DMT1 (9), as defined by our study in Belgrade rats.

Fig. 4. Vascular kinetics of intranasally instilled radioactive ferric iron in homozygous Belgrade b/b, heterozygous iron-deficient ±/b, and heterozygous control ±/b rats. Hematocrit (A), liver nonheme iron (B), brain nonheme iron (C), and body weight (D) are shown (top) for homozygous Belgrade b/b (shaded bar), heterozygote anemic ±/b (solid bar), and heterozygous control +/b (open bar) rats. Bottom: vascular kinetics of radioactive ferric iron uptake in homozygous Belgrade b/b (n = 6, ▲), heterozygote iron-deficient +/b (n = 9, ○), and heterozygous control +/b rats (n = 9, ▼) are shown. Blood samples were collected from the tail vain at 5, 15, 30, 60, 120, and 240 min after instillation, and levels of 59Fe are expressed as percentages of instilled dose. Results shown are the means ± SE. **P < 0.001, *P < 0.05, compared with control +/b rats, †P < 0.001 compared with Belgrade b/b rats, ANOVA.
discussed above. The notion that this pathway is upregulated systemically is reflected in the observed increase of $^{59}$Fe in the red blood cell fraction rather than plasma in iron-deficient rats.

There are alternative explanations for the differences observed in our study. Lower absorption of ferrous iron might reflect greater damage to the epithelial layer relative to the ferric form, and, as a consequence, uptake might be reduced. However, if this explanation were correct, it would be expected that damage should occur equally in relevant tissues of all rats, including control, +/b anemic rats, and b/b rats. The observed differences between ferrous and ferric iron absorption could also reflect relative bioavailability of the different species. Ferrous iron can readily hydrolyze to insoluble oxides, such that absorption via the olfactory pathway may be limited by poor solubility. We reduced iron to the ferrous state with excess ascorbate before instillation, but once the cation is placed in the nasal cavity, its redox state may be altered. Rao et al. (22) studied inhaled ferrous iron and found it bound to mucosal transferrin, suggesting oxidation to Fe(III), which binds to this protein. It is known that ferrous iron binds to factors like mucin (27). Such nonspecific interactions could also reduce the availability. We do not know whether ferrous or ferric iron is the physical form transferred after absorption from the nasal cavity or whether transferrin is involved. Moreover, absorbed iron may be directly transferred to the brain via olfactory neurons (32), or it may follow a vascular pathway across the blood-brain barrier (10). Further study is necessary to refine our knowledge about the molecular details for olfactory iron absorption to the blood and brain. Because olfactory iron absorption is reduced in the Belgrade rat, DMT1 must play a role in the molecular mechanism, but the precise pathway and the ferreductase involved in ferric iron uptake need to be better established. In sum, our observations impart a greater appreciation for the fact that intranasal uptake of iron does occur and is enhanced by iron deficiency. Thus exposure to airborne iron in welding fume, for example, could be associated with higher risk of damage in anemic individuals. Future population studies will be needed to address this issue.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Iron-Deficient +/b rats (n = 9)</th>
<th>Control +/b rats (n = 9)</th>
<th>Belgrade b/b rats (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>3.29 ± 0.756%</td>
<td>2.22 ± 0.667%</td>
<td>3.20 ± 0.718%</td>
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<tr>
<td>Red Blood Cells</td>
<td>+6.30 ± 1.233%</td>
<td>1.15 ± 0.160%</td>
<td>10.75 ± 0.206%</td>
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<tr>
<td>Whole Brain</td>
<td>+0.08 ± 0.012%</td>
<td>0.03 ± 0.013%</td>
<td>0.03 ± 0.008%</td>
</tr>
<tr>
<td>Muscle</td>
<td>+1.32 ± 0.324%</td>
<td>0.68 ± 0.107%</td>
<td>0.35 ± 0.113%</td>
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<tr>
<td>Trachea</td>
<td>0.02 ± 0.003%</td>
<td>0.01 ± 0.002%</td>
<td>0.31 ± 0.217%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.19 ± 0.077%</td>
<td>0.33 ± 0.090%</td>
<td>5.82 ± 3.836%</td>
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<tr>
<td>Lungs</td>
<td>0.25 ± 0.045%</td>
<td>0.09 ± 0.020%</td>
<td>0.28 ± 0.12%</td>
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<td>Spleen</td>
<td>0.97 ± 0.203%</td>
<td>0.28 ± 0.067%</td>
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<td>Heart</td>
<td>0.24 ± 0.044%</td>
<td>0.07 ± 0.012%</td>
<td>0.278 ± 0.147%</td>
</tr>
<tr>
<td>Cecum</td>
<td>+7.44 ± 4.636%</td>
<td>25.62 ± 3.610%</td>
<td>16.90 ± 0.147%</td>
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<tr>
<td>Large intestine</td>
<td>4.16 ± 1.882%</td>
<td>1.81 ± 0.492%</td>
<td>3.46 ± 1.812%</td>
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<tr>
<td>Kidney</td>
<td>0.25 ± 0.044%</td>
<td>0.12 ± 0.023%</td>
<td>0.37 ± 0.186%</td>
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<tr>
<td>Stomach</td>
<td>0.32 ± 0.064%</td>
<td>0.26 ± 0.070%</td>
<td>0.39 ± 0.130%</td>
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<tr>
<td>Stomach content</td>
<td>2.73 ± 1.155%</td>
<td>2.27 ± 0.799%</td>
<td>2.62 ± 0.109%</td>
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<tr>
<td>Jejunum</td>
<td>5.71 ± 1.753%</td>
<td>12.84 ± 4.538%</td>
<td>9.72 ± 3.518%</td>
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<tr>
<td>Liver</td>
<td>+7.60 ± 1.259%</td>
<td>2.10 ± 0.455%</td>
<td>2.38 ± 0.371%</td>
</tr>
<tr>
<td>Sternum</td>
<td>+2.29 ± 0.405%</td>
<td>1.80 ± 0.288%</td>
<td>0.87 ± 0.254%</td>
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Values are means ± SE. Radioactivity is expressed as a percentage of total injected dose for each rat. ANOVA *P < 0.001, †P < 0.05 compared with control +/b rats. ‡P < 0.05 compared with Belgrade b/b rats.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


