Influence of DMT1 and iron status on inflammatory responses in the lung

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Kim J, Molina RM, Donaghey TC, Buckett PD, Brain JD, Wessling-Resnick M. Influence of DMT1 and iron status on inflammatory responses in the lung. Am J Physiol Lung Cell Mol Physiol 300: L659–L665, 2011. First published January 28, 2011; doi:10.1152/ajplung.00343.2010.—Divalent metal transporter 1 (DMT1) is the major iron transporter responsible for dietary iron absorption in the duodenum. It is also essential for iron acquisition necessary for erythropoiesis since it plays a role in transferrin-mediated iron delivery to erythroid cells (9, 16). A glycine-to-arginine missense mutation (G185R) found in both mouse and homozygous Belgrade rats (9, 10). Lack of DMT1 function promotes microcytic hypochromic anemia in these animal models (11, 27, 30).

Many human tissues express DMT1, including intestine, kidney, liver, lung, and olfactory epithelium (3, 17, 32). In rat lungs, DMT1 is expressed in normal airway and alveolar epithelium, especially type II cells (3). DMT1 mRNA expression is also found in bronchus-associated lymphoid tissue adjacent to large airways (3). Certain DMT1 mRNA transcripts contain iron-responsive elements that upregulate expression levels during iron deficiency (5, 15, 16, 18). While body iron status regulates the expression of intestinal and olfactory DMT1, and protein levels correlate with metal transport (32), lung DMT1 mRNA levels do not appear to be associated with lung tissue iron status and/or metal uptake in anemic rats (3, 17, 29). Some studies suggest that DMT1 contributes to uptake and detoxification of iron through regulation of lung mRNA isoforms lacking iron-responsive elements (13, 33). In support of this idea, we have found DMT1 transcripts increase in alveolar macrophages containing iron oxide particles and in nearby epithelial regions (3).

Recent studies of the Belgrade rat model have indicated that low iron status alters pulmonary responses necessary for lung homeostasis in the lung (13, 14). However, the extent to which the low iron status of the Belgrade rat model contributed to the observed effects was not addressed in these studies. It is known that respiratory infections are more common and last longer in iron-deficient children (8). We hypothesized that, under low iron conditions, upregulation of DMT1 may participate in the lung’s inflammatory responses. To address this question, we studied homozygous Belgrade rats carrying nonfunctional DMT1 and their anemia-matched heterozygous littermates to determine how the DMT1 G185R mutation and low iron status alter inflammatory responses following intratracheal instillation of lipopolysaccharide (LPS). Our results show that pulmonary inflammation can be enhanced by the loss of DMT1 function but that these responses are suppressed by low iron status.

MATERIALS AND METHODS

Animals and diets. Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Belgrade rats were maintained on a 12:12-h light-dark cycle and consumed food and water ad libitum. Mating pairs of female heterozygotes (+/b) and male homozygous (bb) Belgrade rats were fed an iron-supplemented diet containing 480–600 mg iron/kg (TD 02385; Harlan Teklad; Table 1). Female +/b rats were fed the iron-supplemented diet throughout pregnancy. At postnatal day 6, litters were cross-fostered to F344 Fischer dams (+/+; Charles River) fed a standard diet containing 220 mg/kg iron (PicoLab 5053; PharmaServ). Three experimental groups were established at the time of weaning: +/b and +/b (control +/b) rats fed the iron-supplemented diet and +/b (anemic +/b) rats fed an iron-deficient diet containing 4–7 mg iron/kg (TD 99397, Harlan Teklad; Table 1) to induce iron-deficient anemia, which serves as an anemia control for bb rats. Belgrade rat genotype was verified by PCR (9).

Analysis of iron status. Hematocrits and liver nonheme iron concentrations were measured as previously described (17). Serum iron was measured as described (6) except that bithiopanthenol sulfate was used as a color reagent. Total iron-binding capacity and transferrin saturation of serum samples were determined based on unsaturated iron-binding capacity (6, 24).

Bronchoalveolar lavage and analysis. Rats (6 wk old) were killed by isoflurane overdose followed by exsanguination for lung lavage and tissue collection. After the trachea was exposed and cannulated,

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the lungs were lavaged 12 times in situ with 3-ml washes of sterile 0.9% Dulbecco’s PBS without calcium or magnesium ions. The first two washes were pooled for biochemical assays. Cells were separated from the supernatant in all washes by centrifugation (400 g at 4°C for 10 min). Total cell counts and hemoglobin measurements were made from the cell pellets. Cell differential counts were determined after Diff-Quik staining (Dade Behring). The supernatant fraction of the first two washes was clarified by centrifugation at 15,000 g for 30 min and used for measurement of enzyme activity and markers for lung injury. Standard spectrophotometric assays were used for lactate dehydrogenase (LDH), myeloperoxidase (MPO), hemoglobin, and albumin (2, 3). Bronchoalveolar lavage (BAL) levels of rat IgM and monocyte chemotactic protein (MCP)-1 were determined by ELISA (Bethyl Laboratory, BD Biosciences). Blood cell morphology was analyzed in Wright’s stained blood smears.

LPS instillation. Another cohort of sibling rats (5–6 wk old) was used to study the role of DMT1 in lung inflammation. LPS from Escherichia coli (O111:B4, L2630; Sigma-Aldrich) was diluted in sterile normal saline before instillation. Rats were anesthetized with vaporized isoflurane and intratracheally instilled with LPS (50 μg/g body wt). Briefly, anesthetized rats were placed on a slanted platform where the larynx was provided by a microscope lamp shining on the neck (4). For LPS-free control groups, normal saline was instilled. Twenty-four hours after administration of LPS or normal saline, rats were similarly anesthetized with vaporized isoflurane and killed by exsanguination, and the liver and blood samples were collected, followed by BAL and analysis.

Statistical analyses. Values reported were expressed as means ± SD. Statistical significance was evaluated using the one-way ANOVA (SPSS, version 17) to compare the differences in parameters among experimental groups. For a pairwise comparison, the Tukey’s test was used as a post hoc analysis, and differences were considered significant at $P < 0.05$.

RESULTS

Characteristics of b/b rats. Physiological and hematological parameters were evaluated in 6-wk-old rats. Body weight was significantly lower in homozygous Belgrade rats (b/b) compared with control or anemic heterozygous (+/b) rats (Table 2). The latter cohort of rats was fed an iron-deficient diet to compare the effects of low iron status on pulmonary inflammation. The hematocrit of b/b rats was greater than that of anemic +/b rats but was significantly lower than control +/b rats. Serum iron status in b/b rats compared with both control and anemic +/b rats was significantly higher and was associated with significantly greater transferrin saturation. Anemic +/b rats displayed lower iron concentrations in serum and liver, whereas b/b rats showed significantly higher liver nonheme iron than control +/b rats. These data are comparable with other studies demonstrating the iron-loading anemia phenotype associated with loss of DMT1 function in the b/b rat (11, 28, 30).

Wright’s staining of blood smears from b/b rats confirmed microcytic hypochromic anemia. In addition, there were a number of cell fragments (schistocytes) with pronounced reticulocyte in +/b rats compared with the control +/b rats (Fig. 1). However, these features were lacking in the anemic +/b blood smears, which showed the microcytic hypochromic anemia induced by dietary iron deficiency.

BAL in b/b rats. Despite the significant differences in iron status, BAL fluid did not contain any detectable iron in all three groups (data not shown). Endogenous biochemical and cellular parameters in BAL of control, anemic, and b/b rats are shown in Fig. 2. There was significantly greater recovery of total cells and macrophages in BAL fluid of the b/b rat compared with control and anemic rats (Fig. 2A). Macrophages were the most abundant cell type, representing ~97% of total lavaged cells in all three groups. Although the numbers of neutrophils, lymphocytes, and eosinophils were greater in b/b than in control +/b and anemic +/b rats (Fig. 2A), the percentage of these cell types relative to the total BAL cell population did not differ among the three groups (data not shown). The b/b rats showed significantly higher levels of LDH and MPO than control or anemic +/b rats (Fig. 2B). Albumin and hemoglobin levels were also significantly elevated in b/b compared with +/b rats (Fig. 2B). Collectively, these data show that b/b rats display mild pulmonary inflammation under resting conditions.

Effects of LPS-mediated lung injury and inflammation. To characterize directly the role of DMT1 and iron status in

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>Diet Components</th>
<th>TD 02385</th>
<th>TD 99397</th>
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</thead>
<tbody>
<tr>
<td>Iron, mg/kg</td>
<td>480-600</td>
<td>4-7</td>
</tr>
<tr>
<td>Casein, g/kg</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>dt-methionine, g/kg</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Sucrose, g/kg</td>
<td>533</td>
<td>545</td>
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<td>Corn starch, g/kg</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Corn oil, g/kg</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix (iron-deficient, 81062), g/kg</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix (AIN-76N, 40077*), g/kg</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Choline bitartrate, g/kg</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant), g/kg</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein, %</td>
<td>17.8</td>
<td>17.7</td>
</tr>
<tr>
<td>Carbohydrate, %</td>
<td>68.7</td>
<td>69.8</td>
</tr>
<tr>
<td>Fat, %</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Energy, kcal/g</td>
<td>3.9</td>
<td>4.0</td>
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</table>

The two diets are based on TD 80396. *Reeves et al. (25).

Table 2. Physiological and hematological characteristics of 6-wk-old rats

<table>
<thead>
<tr>
<th></th>
<th>Control +/b</th>
<th>n</th>
<th>Anemic +/b</th>
<th>n</th>
<th>b/b</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>126.2 ± 24.1</td>
<td>12</td>
<td>118.2 ± 20.5</td>
<td>10</td>
<td>91.5 ± 17.9*‡</td>
<td>13</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>41.4 ± 2.9</td>
<td>12</td>
<td>23.9 ± 4.2†</td>
<td>10</td>
<td>31.5 ± 2.4‡</td>
<td>13</td>
</tr>
<tr>
<td>Serum iron, μg/ml</td>
<td>2.7 ± 0.6</td>
<td>12</td>
<td>0.6 ± 0.2†</td>
<td>10</td>
<td>5.5 ± 0.7‡</td>
<td>13</td>
</tr>
<tr>
<td>TIBC, μg/ml</td>
<td>4.6 ± 0.6</td>
<td>6</td>
<td>5.0 ± 0.3</td>
<td>4</td>
<td>6.1 ± 0.2‡</td>
<td>7</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>62.4 ± 19.4</td>
<td>6</td>
<td>11.1 ± 6.2†</td>
<td>4</td>
<td>99.2 ± 1.1*‡</td>
<td>7</td>
</tr>
<tr>
<td>Liver nonheme iron, μg/g</td>
<td>109 ± 68</td>
<td>12</td>
<td>11 ± 3†</td>
<td>10</td>
<td>174 ± 87*‡</td>
<td>13</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; n, no. of rats. *$P < 0.05$ between control +/b and b/b rats. †$P < 0.05$ between control +/b and anemic +/b rats. ‡$P < 0.05$ between anemic +/b and b/b rats. TIBC, total iron-binding capacity.
pulmonary inflammation, we studied the effects of intratracheally instilled LPS on BAL physiology in the three groups of rats. Exposing rats to LPS did not change body weight or systemic physiological parameters compared with saline-instilled rats (Table 3). As expected, LPS instillation changed BAL physiology in the three groups of rats. Exposing rats to LPS did not change body weight or systemic physiological parameters compared with saline-instilled rats (Table 3). As expected, LPS instillation changed BAL physiology in the three groups of rats.

DISCUSSION

Our group has studied the influence of iron status on pulmonary transport of metals (3, 17, 29) and demonstrated that potential toxicity of instilled metals can be modified by iron status. In this report, we extend these findings to define the role of DMT1 and iron status in pulmonary inflammation. To do so, we performed BAL experiments to compare pulmonary inflammatory responses between homozygous Belgrade rats and their control and anemic heterozygous littermates following intratracheal instillation of saline or LPS. We found that b/b rats display elevated pulmonary inflammation in both resting and LPS-instilled conditions. Because loss of DMT1 activity produces microcytic anemia, we also compared heterozygous control rats with heterozygotes fed an iron-deficient diet to induce microcytic anemia. Pulmonary inflammation appeared to be suppressed in the latter group. These combined results support the concept that DMT1 plays an active role in modulating inflammation in the lung.

DMT1 appears critical for protection from oxidative damage to the lung epithelial surface (13, 14). LPS and inflammatory stimuli like tumor necrosis factor-α and interferon-γ have been demonstrated to elevate DMT1 expression (23, 33, 34). Although DMT1 expression is not correlated with pulmonary absorption of iron and manganese in rats (3), this transport protein may contribute to other lung-associated functions. For example, DMT1 in macrophages could operate at the level of the macrophage phagolysosome such that export of solubilized metal from phagolysosomes might maintain a concentration gradient for dissolution of particles (3, 20). Thus, increased levels of DMT1 may be related to maximizing particle dissolution rates within phagocytic cells but have little to do with metal transport across the air-blood barrier (3). It is unclear why b/b rats have more macrophages in the lung than their littermate +/b rats under resting conditions (Fig. 2A). We speculate that the lack of functional DMT1 in b/b rats is compensated by upregulation of macrophage recruitment to the lungs to protect them from environmental pollutants by optimizing particle phagocytosis and dissolution.

The b/b rats also showed a significant increase in parameters characteristic of inflammation and lung injury, including LDH, MPO, albumin, and hemoglobin, compared with control or anemic +/b rats (Fig. 2B). The increased levels of lung injury markers IgM and MCP-1 in saline-instilled rats, compared with +/b rats, again indicating mild lung injury, as seen in Fig. 2. Following LPS instillation, there were significant increases in all four inflammatory markers compared with the respective saline-instilled groups. Compared with control +/b rats, b/b rats displayed similar enzyme levels (LDH and MPO) in the BAL fluid after LPS instillation (Fig. 4B) but significantly elevated levels of albumin and IgM (Fig. 4C). In contrast, anemic +/b rats exhibited attenuated inflammatory responses after LPS challenge, indicating that elevated inflammatory responses observed in b/b rats are more likely a consequence of defective DMT1 than a result of their anemic condition.
also support these findings (Fig. 4, B and C). In contrast, Ghio et al. (13, 14) showed no change in LDH or protein concentration in BAL fluid from the b/b rats compared with control rats either at the resting condition or after saline instillation. One difference is that we compared a diet-matched heterozygous control group of rats in our study. We also observed an increase in the percentage of neutrophils in b/b rats after LPS instillation, whereas Ghio et al. (13) did not, but this observation might be due to different inflammatory stimuli (oil fly ash vs. LPS). Despite the differences in lung challenges and diets, it is clear from all of these investigations that DMT1 plays an important role in pulmonary inflammatory responses.

The present study further focused on the potential influence of anemia on BAL physiology and the inflammatory response to LPS. Of significant note is the reduced level of MPO activity (Figs. 2B and 4B). Because MPO requires heme as a cofactor, anemia is associated with MPO deficiency (22). It is conceivable that anemic b/b rats, as a result of insufficient heme production, would have a lower MPO level as well. However, b/b rats showed greater MPO levels in BAL than +/b rats (Figs. 2B and 4B). It is possible that elevated neutrophil degranulation could have offset lower MPO production in b/b rats compared with the anemic rats. Another possibility is that neutrophils take up sufficient iron for heme synthesis by a pathway independent of DMT1 function. Serum iron levels and transferrin saturation indicate iron availability in the b/b rat, but without DMT1 there is insufficient hemoglobinization in red blood cells. Overall, inflammatory responses measured in our study were reduced in anemic +/b rats compared with both control +/b and b/b rats.

Table 3. Physiological characteristics of b/b rats after LPS intratracheal instillation

<table>
<thead>
<tr>
<th></th>
<th>Control +/b</th>
<th>n</th>
<th>Anemic +/b</th>
<th>n</th>
<th>b/b</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>98.2 ± 10.9</td>
<td>6</td>
<td>75.5 ± 20.2</td>
<td>7</td>
<td>80.6 ± 29.9</td>
<td>6</td>
</tr>
<tr>
<td>LPS</td>
<td>99.0 ± 8.2</td>
<td>6</td>
<td>72.4 ± 10.8‡</td>
<td>8</td>
<td>81.4 ± 23.3</td>
<td>6</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>38.5 ± 0.6</td>
<td>6</td>
<td>16.8 ± 1.1†</td>
<td>7</td>
<td>29.4 ± 1.9‡</td>
<td>6</td>
</tr>
<tr>
<td>Serum iron, μg/ml</td>
<td>38.2 ± 1.3</td>
<td>6</td>
<td>15.9 ± 1.7†</td>
<td>8</td>
<td>29.9 ± 7.5‡</td>
<td>6</td>
</tr>
<tr>
<td>Liver nonheme iron, μg/g</td>
<td>2.2 ± 0.3</td>
<td>6</td>
<td>0.6 ± 0.2†</td>
<td>7</td>
<td>5.4 ± 1.1‡</td>
<td>6</td>
</tr>
<tr>
<td>LPS</td>
<td>1.8 ± 0.4</td>
<td>6</td>
<td>0.6 ± 0.2†</td>
<td>8</td>
<td>4.7 ± 1.1‡</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3. Physiological characteristics of b/b rats after LPS intratracheal instillation

- Data are presented as means ± SD; n, no. of rats. LPS, lipopolysaccharide. *P < 0.05 between control +/b and b/b rats. †P < 0.05 between control +/b and anemic +/b rats. ‡P < 0.05 between anemic +/b and b/b rats.
This is consistent with evidence that iron deficiency suppresses immune responses (1, 7, 19).

To characterize the neutrophil-macrophage relationship quantitatively after LPS instillation, we compared the ratio of macrophages to neutrophils between b/b and +/b rats. Enhanced numbers of macrophages in b/b rats may result in unwanted inflammatory reactions and could increase lung injury and membrane permeability, which appears to be in part represented by significantly elevated albumin and IgM (Fig. 4C). It is unlikely that these effects were induced by the high iron in the lungs, since tissue iron levels were not different between control +/+ and b/b rats (31). However, it is important to note that serum iron levels are high in b/b rats due to ineffective erythropoiesis. Our study compared b/b with b/+ rats, but whether high iron status of the b/b rat contributes to the observed differences cannot be ascertained. In a rat model of dietary iron overload, we did not observe obvious histological differences, suggesting that high iron status alone would not enhance lung inflammation or injury due to macrophage recruitment (29). However, further studies would be useful to determine to what extent high iron status modifies pulmonary responses to LPS instillation and how DMT1 functions in this setting. It is known that chronic airway infections and lung injury can be associated with elevated iron status (21, 26). Modulation of DMT1 activity in the lung may represent a potential therapeutic target, since it has been suggested that use of iron chelators and nutritional deficiency may be useful in lung disease (12, 21, 26). Further study is necessary to enlighten the mechanistic relationship between DMT1 and lung inflammation and to determine the physiological and toxicological consequences of DMT1 functions in the lung.

GRANTS
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DISCLOSURES
The authors have no conflicting financial interests.

REFERENCES


