A Postweaning Iron-Adequate Diet Following Neonatal Iron Deficiency Affects Iron Homeostasis and Growth in Young Rats1,2

Narasimha V. Hegde,* Gordon L. Jensen, and Erica L. Unger

Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802

Abstract

Iron deficiency is among the most prevalent of nutrient-related diseases worldwide, but the long-term consequences of maternal and neonatal iron deficiency on offspring are not well characterized. We investigated the effects of a postweaning iron-adequate diet following neonatal iron deficiency on the expression of genes involved in iron acquisition and homeostasis. Pregnant rats were fed an iron-adequate diet (0.08 g iron/kg diet) until gestational d 15, at which time they were divided into 2 groups: 1) a control group fed an iron-adequate diet, and 2) an iron-deficient group fed an iron-deficient diet (0.005 g iron/kg diet) through postnatal d (P) 23 (weaning). After weaning, pups from both dietary treatment groups were fed an iron-adequate diet until adulthood (P75). Rat pups that were iron deficient during the neonatal period (IDIA) had reduced weight gain and hemoglobin concentrations and decreased levels of serum, liver, and spleen iron on P75 compared with rats that were iron sufficient throughout early life (IA). IDIA rats developed erythrocytosis during postweaning development. Further, hepatic expression of hepcidin in IDIA rats was 1.4-fold greater than in IA rats, which paralleled an upregulation of IL-1 expression in the serum. Our data suggest that an iron-adequate diet following neonatal iron deficiency induced an inflammatory milieu that affected iron homeostasis and early growth and development. J. Nutr. 141: 386–390, 2011.

Introduction

Iron deficiency and resulting anemia have a worldwide economic impact that is estimated in the billions of dollars (1). An increased requirement of iron during pregnancy and lactation places mothers and infants at high risk of iron deficiency, even in developed countries (2,3). Iron deficiency during infancy and its effects on cognitive and behavioral functions have been well documented; both human and rodent studies have indicated that iron supplementation cannot correct all behavioral and neurochemical deficits associated with deficiencies in iron in early life (4,5). However, the long-term effects of early iron deficiency on iron regulation outside of the brain have received little attention.

Iron regulation occurs primarily through the regulation of hepcidin expression in hepatocytes, and signaling pathways such as Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT), IL6/STAT3, and Bone morphogenic protein (BMP/Smad) appear to play pivotal roles in this process. However, dietary iron-dependent signals and the proteins that mediate these pathways are still being investigated. Iron homeostasis operates through a coordinated regulation between different organs, such as the intestine where dietary iron is absorbed, bone marrow where RBC are produced, liver where excess iron is stored, and spleen where RBC are catabolized and iron is extracted for reuse (6). Iron absorption in the intestine is regulated in response to body iron stores and the erythropoietic requirement. During iron deficiency, iron uptake is enhanced until iron storage is replete. During the period of iron repletion, regulation is thought to operate through iron storage, recycling, and erythropoietic demand (7). Although pathways operating during iron deficiency to increase iron acquisition are less well defined, the involvement of the recently identified peptide hepcidin in iron homeostasis and its regulation by anemia, hypoxia, and inflammation have begun to give us a better understanding about the molecular events that occur during iron deficiency and iron repletion (8–10). A role for hepcidin in iron homeostasis is evidenced by the fact that a complete lack of hepcidin in mice leads to progressive iron accumulation, whereas transgenic animals overexpressing hepcidin in the liver have decreased body iron levels and develop microcytic anemia (11,12).

In rodents, the negative impact of early life iron deficiency on behavior and cognition (13–15), inflammatory colitis (16), and physical growth (17,18) has been well studied. However, studies focused on the consequences of neonatal iron deficiency and subsequent iron repletion are lacking with regard to its long-term impact on iron regulation. In this study, we examined the effect of postweaning iron supplementation following neonatal iron deficiency on the growth of litters and the expression of...
proteins involved in iron homeostasis. We hypothesize that depletion of iron during neonatal life followed by an iron-deficient diet during the postweaning period induces inflammation that influences iron absorption and affects early growth and development.

**Materials and Methods**

**Dietary treatments.** Sprague-Dawley rats for breeding stocks were purchased from Harlan Sprague Dawley. Iron-adequate and iron-deficient diets were prepared in our laboratory following the recipe of the AIN-93G diet with cornstarch as the sole source of carbohydrate and ferric citrate as the iron source (19). The iron-deficient diet contained all components of the iron-adequate diet with the exception of ferric citrate, and iron levels were verified using atomic absorption spectrometry after wet digestion with nitric acid. All rats consumed food and deionized distilled water ad libitum and were housed in a temperature (22 ± 1°C) and humidity (40%) controlled room maintained on a 12:12-h-light/dark cycle.

Female breeder rats were fed a powdered iron-adequate diet (0.08 g iron/kg diet) for 3 wk prior to mating. Pregnant dams continued to be fed an iron-adequate diet until gestational d 15 (G15), at which time they were divided into 2 groups: a control group fed an iron-adequate diet (0.08 g iron/kg diet) and an iron-deficient group fed an iron-deficient diet (0.005 g iron/kg diet) through postnatal d (P) 23 (weaning). After weaning, pups from both dietary groups were fed iron-adequate diet for 7 wk (until P75). This feeding schedule produced 2 groups of experimental rats: 1) always iron adequate (IA) and 2) iron deficient from G15 to P23 and then iron adequate beginning at P23 (IDIA). Litters were culled to P2 or 10 pups/litter. Four litters of rats were generated from 4 different breeding pairs for each diet group, and an equal representation of male and female offspring were selected for analysis at P23 and P75 to control for dam-specific effects on the outcomes (from 3 independent trials). All experimental protocols were in accordance with the NIH Animal Care guidelines and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

**Tissue and blood collection.** IA and IDIA rats were decapitated on P23 or P75 after brief exposure to CO2 and trunk blood was collected. Whole blood was centrifuged at 16,000 × g at 4°C for 15 min and then sera were stored at −80°C. One lobe from each liver and a portion of spleen were quickly dissected and stored in RNA later (Sigma) for total RNA isolation. A second lobe of liver and a portion of spleen were stored in RIPA buffer (Pierce) containing complete mini protease inhibitor cocktail (Roche) for protein analysis. The remaining liver and spleen samples were used to estimate tissue iron content. All samples were stored at −80°C until further processing.

**Liver, spleen, and serum iron determination.** Liver and spleen iron concentrations were determined using a spectrophotometric method modified from Cook (20). Serum iron concentrations and total iron binding capacity were determined using a spectrophotometric method with hemoglobin standard solution according to the manufacturer’s instructions (Sigma Aldrich). Hematocrit was calculated after centrifugation of blood samples in heparinized microcapillary tubes. Blood cell morphology was studied after Giemsa staining of blood smears.

**Serum erythropoietin and IL-1α measurement.** Serum erythropoietin concentrations of IA and IDIA rats were determined by immunoassay (ELISA) using a Quantikine Rat Erythropoietin assay kit (R&D Systems). Expression of IL-1α in the serum of IA and IDIA rats on P75

---

**RESULTS**

**Hemoglobin, hematocrit, serum, liver, and spleen iron concentrations.** Compared with IA rats, hemoglobin concentrations were reduced in IDIA rats on P23 (P < 0.001) as well as on P75 (P < 0.001; Table 1). Hematocrit values were also reduced in IDIA rats on P23 (P < 0.01) compared with IA rats. However, on P75, hematocrit values were elevated in IDIA rats compared with IA rats (P < 0.01; Table 1). Iron deficiency was determined using Proteome Profiler antibody arrays (Rat Cytokine Array Panel A).

**Total RNA isolation, RT, and real-time PCR.** Total RNA from liver tissue was isolated using an RNeasy mini kit (QIAGEN) following the protocol provided by the manufacturer. To eliminate genomic DNA contamination in the RNA preparation, on-column DNase treatment was performed during RNA isolation. RNA quality was examined with standard 260/280-nm spectrophotometer readings and this ratio was maintained > 2.1. One microgram of total RNA was used to prepare cDNA using an RT2 First Strand kit (SABiosciences). The PCR mix (25 μL reaction volume) in triplicate consisted of 12.5 μL iQ SYBR Green Supermix (Bio-Rad), 200 nmol/L primers (hepcidin F- taggaaggaagtgg, hepcidin R- cagaaagcaagcagcagacag), and 1 μL cDNA. PCR reactions were performed with the following cycling conditions: 1 cycle at 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Data were analyzed using the standard ΔΔCt method using β-actin as an internal control.

**Protein isolation and Western-blot analysis.** Liver tissues were homogenized in cold RIPA buffer containing protease inhibitor cocktail (Roche). Cell debris was centrifuged (20,000 × g, 20 min, 4°C) and the total protein content of the extracts was assessed using the BCA protein assay (Thermo Scientific). A total of 20 μg of total protein was loaded onto a 10% SDS-PAGE and separated by electrophoresis. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were blocked using phosphate buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.76 mmol/L KH2PO4, pH 7.4) containing 0.05% Tween 20 and 5% nonfat dried milk. Rabbit polyclonal antibodies to transferrin receptor (TfR)2 and ferritin (Santa Cruz Biotechnology) were used to detect the expression of these proteins in the liver tissue. Respective HRP conjugated secondary antibodies (Sigma Aldrich) were used to identify the protein band of interest using Super Signal West Pico Chemiluminescent substrate (Pierce). The membranes were stripped and reprobed with anti-β-actin antibody (Sigma Aldrich) to confirm equal loading and transfer. Protein expression levels were quantitated by densitometric scanning using a Carestream Molecular Imaging system.

**Statistical method.** Data are presented as means ± SEM. Differences between means were tested for significance using t test probabilities. Differences were considered significant at P < 0.05.

**TABLE 1** Hematology and nonheme iron concentrations of IA and IDIA rats at P23 (weaning) and P75 (adulthood)

<table>
<thead>
<tr>
<th></th>
<th>P23</th>
<th>P75</th>
<th>P23</th>
<th>P75</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>IDIA</td>
<td>IA</td>
<td>IDIA</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>120 ± 6</td>
<td>75 ± 5*</td>
<td>147 ± 23</td>
<td>108 ± 20*</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.34 ± 0.03</td>
<td>0.24 ± 0.05*</td>
<td>0.48 ± 0.02</td>
<td>0.58 ± 0.03*</td>
</tr>
<tr>
<td>Serum iron, μmol/L</td>
<td>28 ± 6.5</td>
<td>13 ± 3.4*</td>
<td>93 ± 8.5</td>
<td>40 ± 4.3*</td>
</tr>
<tr>
<td>Liver iron, μmol/g</td>
<td>0.05 ± 0.04</td>
<td>0.44 ± 0.03*</td>
<td>4.54 ± 0.60</td>
<td>2.60 ± 0.22*</td>
</tr>
<tr>
<td>Spleen iron, μmol/g</td>
<td>2.72 ± 0.30</td>
<td>0.78 ± 0.09*</td>
<td>9.40 ± 0.30</td>
<td>4.80 ± 0.20*</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>25 ± 3</td>
<td>14 ± 2*</td>
<td>93 ± 8</td>
<td>40 ± 3*</td>
</tr>
</tbody>
</table>

*Abbreviations used: IA, iron-adequate diet; IDIA, iron deficiency during neonatal period followed by iron adequate diet; G15, gestational d 15; P23, postnatal d 23; P75, postnatal d 75, TfR2, transferrin receptor 2.

1 Values are means ± SEM, n = 8. *Different from IA on that day, P < 0.05.
during the neonatal period reduced serum iron and liver and spleen iron concentrations in IDIA rats relative to IA rats. On P23, serum iron concentrations were 54% lower \( (P < 0.05) \), liver iron concentrations were 48% lower \( (P < 0.001) \), and spleen iron concentrations were 71% lower \( (P < 0.001) \) in IDIA than in IA rats. On P75, IDIA rats had serum iron concentrations that were 25% lower \( (P < 0.001) \), liver iron concentrations that were 43% lower \( (P < 0.001) \), and spleen iron concentrations that were 49% lower \( (P < 0.05) \) than those in IA rats (Table 1). IDIA rats had transferrin saturations that were 44% and 25% lower \( (P < 0.001) \) on P23 and P75, respectively, compared with IA rats (Table 1).

Blood cell morphological analysis showed that IDIA rats developed erythrocytosis during the postweaning period (Fig. 1). Serum erythropoietin concentrations were elevated in IDIA rats compared with IA rats on P23 \( (P < 0.001; \text{Fig. 2}) \). However, serum erythropoietin was not significantly different between diet groups at P75.

**Growth of rats during the postweaning period.** Body weight was lower in IDIA rats compared with IA rats on P23, P40, P55, and P75 \( (P < 0.05 \text{ for all}; \text{Fig. 3}) \).

**Expression of iron regulatory proteins in liver.** The expression of genes that are indicators of tissue iron status was examined in IA and IDIA rats. In IDIA rats, liver hepcidin mRNA levels were 80% lower than in IA rats on P23 \( (P < 0.001; \text{Fig. 4A}) \). However, by P75, expression of hepcidin in IDIA rats was 1.4-fold greater than that of IA rats \( (P < 0.001; \text{Fig. 4A}) \). Similarly, expression of the inflammatory cytokine IL-1α in IDIA rats was 2.6-fold that of IA rats at P75 \( (P < 0.005; \text{Fig. 4B}) \).

**Discussion**

In this study, we examined the effects of iron deficiency during late gestation and the lactational period on offspring development and iron metabolism in early adulthood. The reduction in dietary iron from G15 through P23 resulted in the development of moderate anemia in dams (hemoglobin levels were 156 g/L in rats receiving the iron-adequate diet and 98 g/L in rats given the iron-deficient diet). Pups nursed by these dams utilize their reserve iron more rapidly and experience iron deficiency during the suckling (neonatal) period. During the neonatal period, rats undergo major changes in their intestine. Solid food consumption begins around P16, leading to a decrease in the consumption of milk. The effects of transitioning from a low-iron diet (rat milk) to a normal-iron diet (nonpurified diet) on the maturation of the rat intestine have been well studied \( (21) \), as has the process of iron absorption in suckling rats \( (22,23) \). However, iron deficiency during the neonatal period and the cellular changes brought about by dietary iron during postweaning development have yet to be examined at the molecular level.

In the current study, 7 wk of iron-adequate diet (postweaning) did not restore normal iron variables in the rats that experienced iron deficiency during the neonatal period. Rat pups from iron-deficient dams (IDIA) had reduced hematocrit levels at weaning, as we would expect during iron deficiency, but by P75, hematocrit values were 20% higher in IDIA rats compared with those in IA rats. This increase in hematocrit may best indicate abnormal erythropoiesis during postweaning development. An elevated hematocrit may be caused by an absolute increase in blood cells, which may be secondary to a decreased amount of oxygen (hypoxia) during iron deficiency or the result of a proliferation of blood forming cells in the bone marrow \( (24) \). Development of erythrocytosis in IDIA rats during postweaning development would complement the observation of increased hematocrit. Several factors such as acute anemia, hypoxia, and chronic inflammatory disorders have been reported to contribute to the pathophysiology of anemia and erythrocytosis \( (25) \). Because iron can increase radical promoting nontransferrin bound iron, development of an inflammatory reaction can be expected either during dietary iron supplementation or during the time of blood transfusion. It has been reported that blood transfusion increases radical promoting nontransferrin bound iron in preterm infants \( (26) \), and RBC transfusion in mice produces harmful effects that are mediated by iron and inflammation \( (27) \). Our findings provide what we think to be the first observation of dietary iron-induced erythrocytosis during postweaning development following neonatal iron deficiency.

Liver, spleen, and serum iron concentrations at P75 were significantly lower in IDIA rats compared with the IA rats, although serum erythropoietin concentrations in IDIA rats were comparable to those in IA rats. Serum cytokine profiling of IA and IDIA rats at P75 indicate an elevated expression of IL-1α in the serum of IDIA rats. This observation suggests that postweaning, the iron-adequate diet induced a proinflammatory reaction in these rats. It has previously been shown that chronic infusion of IL-1 to mice leads to reduced serum iron, hemoglobin, low saturation of transferrin \( (28) \), and the development of

---

**FIGURE 1** Giemsa staining of blood smears from IA and IDIA rats at P23 (weaning) and P75 (adulthood). A representative image is shown for each group, \( n = 6 \).

**FIGURE 2** Serum erythropoietin concentrations from IA and IDIA rats determined by immunoassay (ELISA). Values are means ± SEM, \( n = 8 \). *Different from IA, \( P < 0.001 \).
hypoferremia. This condition was explained by the restricted availability of iron for erythropoiesis. In our study, postweaning dietary iron induced IL-1α in IDIA rats, which explains the reduction in serum iron, hemoglobin, and transferrin saturation values in IDIA rats at P75. In addition, our studies on bone marrow and spleen from IA and IDIA rats have indicated that bone marrow erythropoiesis is suppressed and splenic erythropoiesis is activated in IDIA rats at P75 (data not shown). These data further support the observations of the development of erythrocytosis in IDIA rats during the postweaning period.

To examine the changes in iron regulatory proteins in the liver during postweaning development, hepatic expression of TfR2 and ferritin were examined, because these 2 proteins best indicate body iron status and iron storage. Most iron circulates in the blood bound to transferrin. The binding and release of iron to transferrin is pH dependent, which allows transferrin to deliver iron to cells through receptor-mediated endocytosis via TIR 1 and 2. Expression of TIR2 is largely restricted to the liver and it has been suggested that TIR2 acts primarily as an iron sensor (29). As expected in IDIA rats, expression of TIR2 was elevated on P23 to facilitate iron absorption. A small decrease (20%) in TIR2 expression on P75 in IDIA rats was observed despite a lack of repletion in iron stores in IDIA rats. Ferritin, the iron storage protein, was consistently reduced on P23 and P75 in IDIA rats, reflecting lower iron storage (data not shown).

Hepcidin antimicrobial peptide expressed in the liver inhibits both iron absorption in the small intestine and the release of recycled iron from macrophages. More recently, it was shown that IL-1α, IL-1β, and IL-6 can act on hepatocytes to stimulate hepcidin production (30,31). In the current study, dietary iron deficiency during the neonatal period decreased liver hepcidin expression on P23, consistent with the reported role of hepcidin during iron deficiency (32). However, providing an iron-adequate diet postweaning resulted in a 1.4-fold increase in hepcidin expression in IDIA rats, although liver iron concentrations were 43% less than in IA rats. Inflammatory cytokines such as IL-1 and IL-6 increase hepcidin expression, and macrophage elaboration of IL-6 induces hepcidin expression during inflammation, leading to hypoferremia (33,34). Serum cytokine profiling of IA and IDIA rats at P75 indicated an elevation in the expression of IL-1α in the serum of IDIA rats. This protein array also represented IL-1β and IL-6; however, we did not see any changes in these 2 inflammatory cytokines in the serum of IDIA rats. This observation suggests that the iron-adequate diet provided during the postweaning period induced inflammatory cytokine IL-1α expression; this in turn might have influenced hepcidin expression in IDIA rats.

In the past 10 y, experiments have been conducted to understand the molecular signals affecting iron metabolism by injecting turpentine or LPS to induce inflammation and then investigating how these treatments affect iron homeostasis (35–37). This approach does not provide information on dietary iron-induced signals and, more importantly, iron deficiency-induced abnormalities during early development and the persistent effects that occur later development. Importantly, preterm infants and neonates are thought to be at particular risk of oxidative stress because of incomplete maturation of the antioxidant defenses (38–40). Overall, our study found that providing an iron-adequate diet following neonatal iron deficiency may alter iron homeostasis by inducing the expression of the inflammatory cytokine IL-1α to modulate the expression of iron regulatory proteins, and in turn, these effects may alter early growth and development. This is to our knowledge the first report suggesting that the provision of postweaning dietary iron may induce erythrocytosis. The mechanisms by which dietary...
iron supplementation following neonatal iron deficiency coordinates erythropoietic demand and iron absorption remain to be elucidated.

Acknowledgments
We thank Dr. Catharine Ross for helpful discussions and critical reading of the manuscript. N.V.H. designed the experiments, conducted the research, and wrote the manuscript; E.L.U. and G.L.J. helped in the interpretation of the data and edited the manuscript; and N.V.H. had primary responsibility of the final content. All authors read and approved the final manuscript.

Literature Cited
5. Felt BT, Beard JL, Schallert T, Shao J, Aldridge JW, Connor JR. Early iron supplementation following neonatal iron deficiency coor-