Reduced Expression of Ferroportin-1 Mediates Hyporesponsiveness of Suckling Rats to Stimuli That Reduce Iron Absorption

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BACKGROUND & AIMS: Suckling mammals absorb high levels of iron to support their rapid growth. In adults, iron absorption is controlled by systemic signals that alter expression of the iron-regulatory hormone hepcidin. We investigated whether hepcidin and absorption respond appropriately to systemic stimuli during suckling. **METHODS:** In Sprague–Dawley rats, iron levels increased following administration of iron dextran, and inflammation was induced with lipopolysaccharide. Gene expression was measured by quantitative reverse-transcription polymerase chain reaction; protein levels were measured by immunoblot analyses. Iron absorption was determined based on retention of an oral dose of ^{59}Fe. **RESULTS:** Iron absorption was high during suckling and reduced to adult levels upon weaning. In response to iron dextran or lipopolysaccharide, iron absorption in adults decreased substantially, but, in suckling animals, the changes were minimal. Despite this, expression of hepcidin messenger RNA was strongly induced by each agent, before and after weaning. The hyporesponsiveness of iron absorption to increased levels of hepcidin during suckling correlated with reduced or absent duodenal expression of ferroportin 1 (Fpn1), normally a hepcidin target. Fpn1 expression was robust in adults. Predominance of the Fpn1A splice variant, which is under iron-dependent translational control, accounts for the low level of Fpn1 in the iron-deficient intestine of suckling rats. **CONCLUSIONS: Iron absorption during suckling is largely refractory to changes in expression of the systemic iron regulator hepcidin, and this in turn reflects limited expression of Fpn1 protein in the small intestine. Iron absorption is therefore not always controlled by hepcidin.**

Keywords: Development; Iron Homeostasis; Nutrition; Infant.

BASIC AND TRANSLATIONAL AT

An adequate supply of iron is essential for good health because this trace element plays a critical role in a wide range of biologic processes. Iron demand is especially high during infancy because of rapid growth and the expansion of the red cell compartment. Consequently, an adequate iron supply during this period is critical for normal development. Iron deficiency at this time of rapid neurodevelopment may have lifelong detrimental effects.[1] This high iron demand is met from a number of sources, including iron stored in the liver in the latter stages of gestation, the catabolism of fetal hemoglobin, and the absorption of iron from the diet. Intestinal iron absorption during suckling is extremely efficient[2] to enable the infant to scavenge as much iron as possible from breast milk, which has a relatively low iron content (from 0.5 μg/mL in humans and dairy animals to 13.5 μg/mL in rats[3]). Around the time of weaning, iron absorption drops substantially to adult levels.[4–6]

How iron traverses the intestine during suckling is poorly understood. Early studies suggested that iron absorption in neonates is a specific, saturable process and is largely restricted to the proximal small intestine,[4,6] ie, characteristics similar to that of iron absorption in adults. However, it has also been suggested that the general high permeability of the small intestine during suckling may contribute to iron absorption, ie, a nonspecific process.[7] We have recently shown that the distal small intestine makes a proportionally larger contribution to iron absorption during suckling than it does postweaning,[6] and this is the region where the highest permeability of the intestine is seen.[8] However, quantitatively, most absorption still occurs in the proximal small intestine.[6] Whatever the mechanism, a number of studies have shown that, in both rodents and humans, iron absorption during suckling is relatively refractory to stimuli, which would reduce absorption in adults.[7,9] In humans, iron supplementation of 6-month-old infants does not lead to a reduction in absorption,[10] whereas, as in adults, at 9 months, iron supplementation does suppress absorption.[11] Similar data have been obtained in rats where 10-day-old suckling animals show little response to an increased body iron load, whereas 20-day-old animals (around the time of weaning) respond by a reduction in the expression of iron transport proteins in the gut.[9]

In adults, the basic components of the pathway by which non-heme iron is absorbed are relatively well defined.[12] Ferric iron in the diet must first be reduced to the ferrous form before it can be utilized. A candidate reductase on the apical or brush border membrane is duodenal cytochrome b. The transport of ferrous iron across the brush border membrane is mediated by divalent metal-ion

Abbreviations used in this paper: BTF3, basic transcription factor 3; DcytB, duodenal cytochrome B; DMT1, divalent metal-ion transporter 1; Fpn1, ferroportin1; IRE, iron responsive element; IRP, iron regulatory protein; LPS, lipopolysacchride; mRNA, messenger RNA; UTR, untranslated region.

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transporter 1 (DMT1). Within the enterocyte, iron is either stored in ferritin or transported across the basolateral membrane by another ferrous iron transporter, ferroportin1 (FPN1). The efficient basolateral efflux of iron also requires an iron oxidase known as *hephaestin*. It is basolateral transport that is rate limiting for iron absorption, and this step is also the target of systemic signals for the regulation of absorption. These signals direct the expression of the liver-derived peptide hepcidin. Hepcidin in turn binds to ferroportin, leading to its internalization and degradation and thereby reducing absorption.[13]

The goal of this study was to determine whether the hepcidin-ferroportin axis is functioning correctly during suckling. We have investigated the mechanism of high iron absorption in suckling rats and in particular how absorption responds to stimuli that repress iron absorption in normal adults. Hepcidin expression increased in response to iron loading and an inflammatory stimulus in both suckling and weaned animals, but only in the older animals did these stimuli decrease iron absorption. The hyporesponsiveness of absorption in the suckling animals can be explained by the demonstration that Fpn1, the target of hepcidin, was either absent or at very low levels during this period. This in turn reflects the pattern of expression of *Fpn1* messenger RNA (mRNA) splice variants in the intestine during suckling, which favors low Fpn1 synthesis in the iron-deficient conditions of the neonatal intestine. These results indicate that the mechanism of iron absorption during suckling is likely to be multifactorial and, at least in younger neonates, that the transport of iron appears to be largely Fpn1 independent.

Materials and Methods

Animals and Treatments

Sprague–Dawley rats were used for all experiments. The rats were maintained on a standard rodent pellet diet (Norco Stockfeeds, South Lismore, Australia; iron content, 160 mg/kg) and were allowed unlimited access to food and deionized water. Animals were weaned at 21 days unless noted otherwise. Some animals were iron loaded by a single injection of 0.3 mg/g iron-dextran (intraperitoneally; Sigma-Aldrich, Sydney, Australia) and killed 4 days later. Inflammation was induced by the injection of lipopolysaccharide (LPS) (0.1 mg/kg, intraperitoneally; Sigma-Aldrich), and the rats were processed for analysis 6 or 10 hours later for *Hepcidin antimicrobial peptide* (*Hamp*) or absorption measurements, respectively. Dexamethasone (0.4 μg/g body weight/day, intraperitoneally; Sigma-Aldrich) was injected for 3 consecutive days, and animals were killed 24 hours after the last injection. Duodenal enterocytes were isolated as previously described,[14] and enterocytes and liver tissue were snap frozen in liquid nitrogen and stored prior to analysis. All experiments described in this study were approved by the Queensland Institute of Medical Research Animal Ethics Committee.

Evaluation of Iron Absorption and Non-Heme Liver Iron

Whole animal absorption measurements were carried out by giving rats an oral dose of ^{59}Fe followed by whole body counting as previously described.[14] Liver tissue was dried at 110°C, and the tissue non-heme iron content was determined colorimetrically as previously described.[15]

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from duodenal enterocytes and liver using TRIzol reagent (Invitrogen, Melbourne, Australia) as per the manufacturer's instructions. Complementary DNA was synthesized using an oligo(dT) primer and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Real-time polymerase chain reaction was performed using LightCycler 480 SYBR Green I Master Mix (Roche, Sydney, Australia) in an LC480 machine (Roche). The data were analyzed by the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta\ C_T}$) and were normalized to basic transcription factor 3 (BTF3). The primers used were as follows: *Hamp1:* forward, GCTGCCTGTCTCCTGCTTCT; reverse, CTGCAGAGCCGTAGTCTGTCTCGTC. *BTF3*: forward, TGGCAGCAAACACCTTCACC; reverse, AGCTTCAGCCAGTCTCCTCAAAC. *Fpn1A:* forward, AAAGAAGACCCCGGTGGCAGC; reverse, GGCCAAGGTAGAGGAGGAATTT. *Fpn1B*: forward, GTTGGTTGGAGTTTCAATGTTG; reverse, GGCCAAGGTAGAGGAGGAATTT.

Western Blot Analysis

Protein was extracted from duodenal enterocytes, and the expression of Fpn1 was determined by Western blotting as described previously.[14,16] Polyclonal antibodies to Fpn1 were raised in rabbits against the peptide CGPDAKEVRKENQANTSVV that corresponds to the C-terminal amino acids 553–571 of the human protein and 68% identical to the rat sequence. Two commercially available anti-Fpn1 antibodies, one raised to the largest extramembrane loop of the human protein and 100% identical to rat sequence (No. ab58695; Abcam, Cambridge, UK) and the other to the C-terminus of murine Fpn1, which is 90% identical to rat (No. MTP11-A; Alpha Diagnostic International, San Antonio, TX), were also used. A rabbit antiactin antibody (Sigma-Aldrich) was used as a loading control.

Statistical Analysis

All values are expressed as mean ± standard error of mean. Statistical differences between means were calculated with PASW Statistics 17.0 (SPSS Inc, Chicago, IL) by using the analysis of variance with Tukey's post hoc test.

Results

Iron Absorption During Suckling and After Weaning

To confirm that iron absorption was showing the expected pattern during the suckling-weaning transition,