Elevated iron absorption in the neonatal rat reflects high expression of iron transport genes in the distal alimentary tract

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Submitted 20 December 2006; accepted in final form 5 July 2007

Frazer DM, Wilkins SJ, Anderson GJ. Elevated iron absorption in the neonatal rat reflects high expression of iron transport genes in the distal alimentary tract. Am J Physiol Gastrointest Liver Physiol 293: G525–G531, 2007. First published July 12, 2007; doi:10.1152/ajpgi.00579.2006.—Intestinal iron absorption is extremely high in neonatal mammals but falls rapidly to adult levels following weaning. The aim of this study was to investigate the molecular basis of this elevated neonatal absorption using the rat as an experimental model. RNA was extracted from various sections of the intestine of 10-, 15-, 20-, 25-, and 30-day-old rats and the expression of the genes encoding DMT1 (Slc11a2), ferroportin (Slc40a1), Cybrd1 (Cybrd1), and hephaestin (hephaestin) determined by ribonuclease protection assay. The hepatic expression of Hamp was studied at the same ages. Iron absorption was examined by following 59Fe uptake in both whole animals and in isolated intestinal loops. Slc11a2, Slc40a1, and Cybrd1 mRNAs were highly expressed in all regions of the small intestine and colon studied in suckling rats. However, after weaning, when iron absorption declined significantly, strong expression was retained only in the duodenum. No change in hephaestin mRNA occurred in any part of the digestive tract. In the distal small intestine and colon, Slc40a1 expression most closely followed the change in absorption that occurred after weaning. Hamp expression was low during the neonatal period and increased to adult levels following weaning. Our results suggest that the distal small intestine and colon contribute significantly to the high intestinal iron absorption seen in neonatal animals and that this reflects increased expression of the iron transporters, particularly Slc40a1.

Although iron is an important nutrient at any stage in life, it is particularly crucial for the period of rapid growth and development that occurs in infancy. Studies have shown that iron deficiency during this period can have long-lasting, detrimental effects on the central nervous system that cannot be corrected by subsequent iron treatment (22, 33). During much of this period, the body's iron requirements are provided solely by breast milk, which is relatively iron deficient (30). Despite this, studies in humans have shown that exclusive breastfeeding is sufficient to maintain an adequate level of body iron stores for at least the first 6 mo of life (35). This suggests that the limited supply of iron provided by breast milk must be very efficiently absorbed by the neonatal intestine.

Studies in humans and experimental animals have confirmed that intestinal iron absorption is extremely high in neonatal mammals and that it shows little or no regulation in response to parenteral iron administration (9, 14, 34). This high absorption, which can represent up to 100% of an ingested iron test dose, persists until weaning but thereafter decreases to adult levels (2, 9). This dramatic change in absorption occurs despite the absence of significant changes in body iron stores (2). The decrease can also be induced by the premature maturation of the neonatal intestine by early weaning or corticosteroid treatment (9, 16). These studies indicate that the high iron absorption during the neonatal period is due to intrinsic characteristics of the intestine rather than an increase in the bioavailability of iron in breast milk.

Recent advances in our understanding of how iron transverses the adult mammalian gut may provide insight into how iron is absorbed in the neonatal animal. The molecular machinery responsible for the intestinal absorption of dietary iron in adult mammals has been extensively studied in recent years. Nonheme iron in the lumen of the intestine is thought to be reduced to the ferrous form by cytochrome b reductase 1 (Cybrd1) on the brush border of the mature villus enterocytes of the proximal small intestine (23). The iron is then transported across the brush border and into the enterocyte by the ferrous iron transporter divalent metal-ion transporter 1 (DMT1), also known as solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2, Slc11a2 (10, 15). Iron is transferred across the basolateral membrane of the enterocyte and into the body by the membrane exporter ferroportin (solute carrier family 40, member 1, Slc40a1) (1, 3, 24). Basolateral transfer also requires the ferroxidase hephaestin although the precise role of this molecule is still unknown (38). The amount of iron absorbed is regulated by hepcidin (encoded by the hepcidin antimicrobial peptide or Hamp gene), a peptide secreted by the liver into the circulation (27, 31). Hepcidin is an inhibitor of iron absorption and appears to act by binding to ferroportin on the basolateral membrane and causing the iron exporter to be internalized and degraded (26).

Although these molecules appear to play important roles in intestinal iron absorption in adult mammals, their role in neonatal iron absorption is unclear. Recent studies have examined DMT1 and ferroportin expression in the duodenum of young rats and have reported little change in expression around the time of weaning and higher expression of both genes in the month following weaning (20, 21). These results suggest that duodenal DMT1 and ferroportin do not contribute to the high intestinal iron absorption seen in neonatal animals. To investigate this further, we have examined the expression of iron transport genes along the entire alimentary canal of the neonatal rat and documented the changes that occur during weaning. Iron absorption and the expression of iron transport genes were higher in the duodenum of neonatal rats than in adult...
animals, but this alone was insufficient to account for the very high iron absorption associated with suckling. However, there was significant expression of Slc11a2, Cybrd1, and Slc40a1 mRNAs in the distal small intestine and colon during the neonatal period. In postweaning animals the expression of these genes is negligible. The increased expression of these iron transport molecules combined with the much larger surface area of the distal small intestine and colon relative to the duodenum appears sufficient to account for much of the high iron absorption observed in neonates. These data support the proposal that the distal small intestine plays an important role in iron absorption during the neonatal period.

MATERIALS AND METHODS

Animals and tissue collection. Pregnant female Sprague-Dawley rats were maintained on a normal pellet diet and given unlimited access to tap water. Following birth, the pups were suckled by the mother until they were 21 days old, at which time the mothers were removed from the cage and the pups weaned onto a standard pellet diet (370 mg iron/kg) (Norco Stockfeeds, South Lismore, Australia). At various ages the pups were either euthanized for tissue collection or used for iron absorption measurements (see Intestinal iron absorption). For tissue collection, animals were anesthetized with 44 mg/kg ketamine (Mavlab, Slacks Creek, Australia) and 8 mg/kg xylazine (Troy Laboratories, Smithfield, Australia) and killed by cervical dislocation. The intestine was removed and sections taken from the duodenum, midjejunum (midpoint of the small intestine), and the ileum. The entire colon was also removed. The sections were cut longitudinally and the enterocytes removed by gentle scraping using a scalpel blade before being snap frozen in liquid nitrogen. A sample of liver tissue was also excised and snap frozen. All experiments described in this study were approved by the Queensland Institute of Medical Research Animal Ethics Committee.

Intestinal iron absorption. Intestinal iron absorption was measured using either intact animals or intestinal loops, depending on the experiment. Whole animal absorptions were carried out by giving rats an oral dose of $^{59}$Fe followed by whole body counting as previously described (13). Intestinal loops were used to determine the amount of iron absorbed from (1) the duodenum (pylorus to the ligament of Treitz), (2) the 5 cm immediately proximal to the cecum, and (3) the entire colon of 15- and 25-day-old rats. Rats were anesthetized as described above and a midline incision made in the abdomen. The appropriate section of the alimentary canal was exposed and three loose ligatures tied around it, two at the proximal end and the other at the distal end, taking care not to inadvertently ligate any blood vessels in the region. An incision was made proximal to the upper ligature, a cannula inserted, and the upper ligature tightened. A second incision was made distal to the lower ligature. The intestinal segment was flushed with $\pm 5$ ml of saline (prewarmed to 37°C) injected through the cannula. The lower ligature was then tightened to seal off the distal end of the segment. The loop was infused with 100 µl of iron solution (250 µM FeNTA$_3$, 125 mM NaCl, 3.5 mM KCl, 16 mM HEPES, pH 7.5) containing 20 µCi/ml $^{59}$FeCl$_3$ (Amersham Biosciences, Buckingham, UK), followed by 150 µl of saline to flush the residual solution from the cannula. The second, lower ligature at the proximal end of the loop was tightened to seal off the intestinal segment and the cannula removed. The abdomen of the animal was covered in damp gauze to prevent drying and continually moistened with prewarmed saline.

Thirty minutes after the administration of the test solution, the animal was killed by cervical dislocation and the duodenal loop carefully excised. The radioactivity in the tied off segment containing the radioactive solution and the radioactivity in the carcass were counted separately using a Ram DA counter with PM-11 tube (Rotem Industries, Arava, Israel) at a distance of 10 cm. The intestinal segment was then rinsed, dried in an oven at 110°C overnight, and weighed. The iron absorbed by the animal was calculated by expressing the amount of radioactivity in the carcass as a percentage of the total radioactivity injected (carcass + tied off segment). The amount of iron absorbed was then calculated by assuming an initial amount of 25 pmol/100 µl dose and this was divided by the dry weight of the segment to get a final value of picomoles of iron absorbed per milligram dry weight of tissue.

Analysis of mRNA expression. Total RNA was extracted from tissue samples by using TRIzol reagent (Invitrogen, Melbourne, Australia) per the manufacturer’s instructions. To measure the levels of mRNAs encoding various proteins involved in iron homeostasis, ribonuclease protection assays (RPAs) were performed as previously described (11) using 5 µg of total RNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used for normalization. The riboprobes used corresponded to the following cDNA sequences (the information in parentheses indicates the section of cDNA used and the Genbank accession number): Hamp (nt1-218, AF344155), Slc11a2 (nt1413-1628, AF029757), Cybrd1 (nt20-259, AF558425), hephaestin (nt1360-1573, AF246120), Slc40a1 (nt1190-1365, U76714), Gapdh (nt536-691, AF106860).

Statistical analysis. All experimental groups contained three to six rats, and values are expressed as means ± SD. Statistical differences between means were calculated by Student’s t-test for comparisons between two groups or by analysis of variance followed by Tukey’s post hoc testing when three or more groups were compared by use of SPSS software (SPSS Australasia, North Sydney, Australia). The results were considered significant at a P value of <0.05.

RESULTS

Whole body iron absorption in neonatal rats. Intestinal iron absorption, as determined using whole animals, was extremely high in 10- and 15-day-old pups (87.0 ± 2.2 and 84.1 ± 4.2% of the test dose of iron, respectively) but decreased dramatically to 50.6 ± 4.9% at 20 days (Fig. 1). By the time the animals were 25 days old, absorption had dropped to a level similar to that in adult animals (7.8 ± 1.7 vs. 10.6 ± 3.5%). This absorption pattern confirms previous reports showing extremely high iron absorption during the suckling period followed by a rapid drop to adult levels at weaning (2).
Intestinal gene expression during the neonatal period. In adults, the majority of dietary iron absorption occurs in the proximal small intestine (7), but the distal small intestine and colon are also capable of absorbing iron in small amounts (3, 17). To understand the basis of the elevated iron absorption associated with the neonatal period, we therefore studied both the entire small intestine and colon. The expression of iron metabolism genes in the small intestine and colon of the rat was determined by RPA before and after weaning. Representative RPAs are shown in Fig. 2, A and B and the quantitation of gene expression is shown in Figs. 2, C–F. The brush border uptake components Slc11a2 and Cybrd1 were expressed at high levels in the duodenum in 15- and 20-day-old animals but not in 10-day-old rats (Fig. 2, A, C, and D). The expression of both molecules then decreased dramatically to adult levels by day 25. Longer exposure times were needed to detect the expression of Slc11a2 and Cybrd1 in the distal gastrointestinal tract; however, the expression of both molecules was higher in the distal small intestine and colon of 10- and 15-day-old animals than it was in older animals. Very little expression of either of these genes was seen at other time points in these tissues. The non-iron response element (IRE) splice variant of Slc11a2 was expressed at very low levels in all tissues and at all time points and was not analyzed further.

The basolateral transporter Slc40a1 and the ferroxidase hephaestin showed a different expression pattern in the duodenum to that of Slc11a2 and Cybrd1. As can be seen in Fig. 2, B, E, and F, no significant changes in the expression of Slc40a1 or hephaestin were observed at any of the ages examined in this region of the intestine. This was also the case with the expression of hephaestin in the distal small intestine and colon. In contrast, the expression of Slc40a1 was increased in the distal small intestine and colon of 10- and 15-day-old animals but not in older rats. Particularly high expression was seen in the colon of 10-day-old animals. As with Slc11a2 and Cybrd1, the expression of Slc40a1 in the distal gastrointestinal tract decreased to adult levels at the time of weaning.

Iron absorption along the length of the gastrointestinal tract during the neonatal period. The increase in Cybrd1, Slc11a2, and Slc40a1 expression in the distal small intestine and colon prior to weaning prompted us to examine the absorption of iron in separate sections of the alimentary canal. Absorption in the duodenum was \( \sim 3.3\)-fold higher at day 15 than day 25 (Fig. 3). This was similar to the difference seen in the distal small intestine with an approximately fourfold higher absorption seen in the 15-day-old rats compared with the 25-day-old animals, although the level of absorption was much lower in the distal small intestine than the duodenum. Surprisingly, the biggest change was seen in the colon with absorption at day 15 being 24 times higher than that at day 25. In fact, the level of absorption in the colon of 15-day-old animals exceeded that of the duodenum in 25-day-old rats.

Hepatic Hamp mRNA expression during the neonatal period. To further investigate the regulation of iron absorption during the neonatal period, we examined the expression of the mRNA encoding the iron regulatory peptide hepcidin in the liver of rats at various ages. As has been shown previously (28), Hamp expression was very low during the neonatal period, being detectable only after much longer exposures in 15-day-old animals (Fig. 4). Hamp expression increased rapidly after weaning to reach a level \( \sim 210\)-fold higher than that seen in 15-day-old rats by day 25. Adult rats had higher expression still, with Hamp expression reaching a level almost 500 times higher than that of 15-day-old rats.

**DISCUSSION**

An adequate supply of iron during infancy is crucial for the proper growth and development of the body (22, 33). The supply of iron to the suckling mammal is maintained by an adaptation of the neonatal intestine that allows the absorption of much higher amounts of iron to maximize absorption from breast milk (9, 14). The molecular basis of this very high absorption has been the focus of the present study.

Several explanations for the high iron absorption during the neonatal period have been proposed. One involves the high capacity of the immature intestine to take up large molecules, such as antibodies, by pinocytosis (4). This pinocytic activity declines with intestinal maturation, leading to the suggestion that the nonspecific uptake of iron by pinocytosis may explain the increased iron absorption in the neonatal animal (9). Several observations, however, suggest that this is not the case. Firstly, the absorption of increasing doses of oral iron indicates that the mechanism of uptake is saturable in neonates (14). Secondly, the ability of the immature proximal intestine to exclude \( { }^{51}\text{Cr-EDTA} \), a nonabsorbable marker, suggests that, at least in this portion of the gut, nonspecific uptake is not occurring (37). Therefore, the increase in iron uptake in the neonate is likely to be the result of stimulation of a specific uptake pathway.

The milk protein lactoferrin also has been suggested to play a role in neonatal iron absorption. Lactoferrin is a member of the transferrin family of proteins and is the major iron binding protein in human milk (18). The discovery of a receptor for lactoferrin on the brush border of the small intestine prompted the suggestion that it may be responsible for the high rate of neonatal iron absorption (19). This is unlikely, however, because the high absorption of orally administered radioiron by these animals does not require the presence of lactoferrin (2, 9, 14). Indeed, a recent study showing that lactoferrin knockout mice develop normally and show no sign of iron deficiency during the suckling period also argues against a role for lactoferrin in neonatal iron absorption, although absorption was not directly measured in this case (39). Lactoferrin has been demonstrated to have a bacteriostatic effect (8, 32), and this may be its primary role in milk.

By measuring the amount of iron absorbed in rats before and after weaning, we have confirmed previous reports (2, 9) showing an extremely high level of intestinal iron absorption during the suckling period in rats. Weaning produced a dramatic decrease in the amount of iron absorbed by the intestine, with absorption falling to normal adult levels by day 25. Gene expression analysis revealed increased levels of Slc11a2 and Cybrd1 in the duodenum of 15- and 20-day-old animals compared with adults, but not in 10-day-old rats. However, this expression pattern did not correlate with the changes in iron transport that occurred, because absorption was also high in 10-day-old animals and had declined significantly by day 20. Because both Slc11a2 and Cybrd1 messages are known to be highly responsive to dietary iron levels (12), this expression pattern may simply reflect a decrease in breast milk iron.
Fig. 2. Expression of iron transport genes in the intestine of rats before and after weaning. Total RNA was extracted from gut samples and gene expression determined by ribonuclease protection assay (RPA) using 5 μg of RNA. Representative gels are shown for Slc11a2 and Cybrd1 (A) and for Slc40a1 and hephaestin (B). The age of the animals in days and the section of gut analyzed are shown at the top of each gel. The exposure time is shown at the bottom of each gel for Slc11a2 and Cybrd1. In the case of Slc40a1 and hephaestin, all gels were exposed to film for 3 h. Band intensities were quantitated by densitometry, corrected for loading using Gapdh as a control, and graphed as a proportion of Gapdh (C–F). Quantitation of Slc11a2 was carried out on the Slc11a2 iron response element (IRE) transcript only, because the expression of Slc11a2 (non-IRE) was negligible in the tissues studied. Data represent means ± SD of 3 animals. Statistical significance is shown relative to the 25-day-old animals (C–F) and the P value indicated at the top of each column.
content toward the end of the suckling period, as has been described in humans (36).

Unexpectedly, the analysis of Slc40a1 expression showed very little change in message level in the duodenum around the time of weaning despite a 3.3-fold decrease in iron absorption in this part of the small intestine over this time. As ferroportin is thought to mediate the rate limiting step in iron absorption, any change in absorption would be expected to involve a change in the expression of this molecule. A possible explanation for this comes from the expression pattern of hepcidin during this period. As hepcidin binds to ferroportin and leads to the iron exporter being internalized and degraded (26), the increase in hamp expression seen after weaning would decrease the amount of functional ferroportin protein on the basolateral surface of duodenal enterocytes and so decrease absorption despite no change in Slc40a1 message levels. Duodenal hephaestin expression did not change throughout the study, although this is not unexpected as hephaestin is not significantly affected by changes in iron absorption (11).

Further analysis of gene expression revealed an increase in Slc40a1, Slc11a2, and Cyb5r1 mRNAs in the distal small intestine and colon of 10- and 15-day-old animals (Fig. 2), a pattern that correlated with the high intestinal iron absorption that occurred at this time (Fig. 1). The expression of these genes decreased to adult levels by day 25 in the distal small intestine and by day 20 in the colon. Subsequent analysis of iron absorption in the distal small intestine and colon using in situ loops revealed 4- and 24-fold higher absorption, respectively, in 15-day-old animals compared with those at day 25, although the changes in the distal small intestine did not reach statistical significance. While the amount of iron absorbed from the distal small intestine and colon was lower than that absorbed from the duodenum, studies have shown that the combined surface area of the jejunum, ileum, and colon makes up ~87% of the total surface area of the intestine (29). When combined with the slower transit rates in the distal gastrointestinal tract, particularly in the colon, it is feasible that the additional iron absorption that occurs in this region during the suckling period could explain the extremely high iron absorption observed in neonatal animals. Supporting this is our observation that the increased iron absorption in the neonatal duodenum relative to the postweaning duodenum is alone unable to account for the high absorption of neonates. If the total body iron absorption seen at day 25 (7.8%) is multiplied by the increase in duodenal absorption seen in the isolated duodenum at day 15 (3.3-fold), total body iron absorption would only increase to ~25%. This is far lower than the 84.7% absorption observed at day 15 and supports a major role for the distal alimentary canal in iron absorption during the suckling period.

The data in Fig. 2 show that the expression of Slc40a1 in the colon progressively decreases with age, reaching adult levels by day 20. A similar expression pattern is shown by molecules involved in the absorption of other nutrients (5). This differs, however, from the expression pattern observed in the jejunum and ileum where Slc40a1 mRNA expression levels are high on days 10 and 15, are at an intermediate level on day 20, and fall to adult levels by day 25. These differences are consistent with the rate of maturation of each tissue. The maturation of the colon to the adult phenotype is complete at the start of the weaning period whereas the small intestine only begins to mature at this time (25).

The changes in whole body iron absorption seen in Fig. 1 are consistent with the observed molecular changes. Absorption was high on days 10 and 15 and reduced to adult levels by day 25. The intermediate level of absorption seen on day 20 was significantly different from all other days, suggesting that it was not caused by interanimal variation in the time required to reach the postweaning phenotype, but truly reflected an intermediate level of absorption. The strong reduction in Slc40a1 expression in the colon and partial reduction in the small intestine could account for the absorption decrease. As maturation of the duodenum did not affect Slc40a1 expression, we anticipate that duodenal iron transport would remain high at day 20. The further drop in absorption to adult levels at day 25

Fig. 3. Iron absorption from intestinal loops in 15- and 25-day-old rats. Intestinal iron absorption was determined in 15- (shaded bars) and 25-day-old (solid bars) rats by using in situ intestinal loops from the duodenum, distal small intestine, and colon. Absorption is presented as the percentage of total radioactivity (carcass + duodenum) transferred to the carcass (carcass radioactivity). The data represent means ± SD of 4–5 animals and the P value for each pair is shown at the top of each column.

Fig. 4. Expression of Hamp in the liver of rats before and after weaning. Total RNA was extracted from liver samples and gene expression was determined by RPA using 5 μg of RNA. A representative gel is shown. Band intensities were quantitated by densitometry, corrected for loading using Gapdh as a control, and graphed as a proportion of Gapdh. The age of the animals in days is shown at the bottom of the figure. Data represent means ± SD of 3–5 animals. Statistical significance is shown relative to the 25-day-old rat and the P value indicated at the top of each column.
is consistent with the increased hepatic expression of *Hamp* at this time, which would inhibit Slc40a1 protein function.

Although it is possible that other, as yet uncharacterized, transport systems may also play a role in the increased iron absorption during the neonatal period, the data presented here suggest that changes in the spatial and temporal expression of molecules used by the adult intestine are able to explain the high absorption in neonates. The decrease in iron transporter expression in the distal small intestine following weaning coincides with the maturation of the intestine. Many other changes also occur at this time, such as an increase in cell proliferation, an increase in the number of crypts per villus, and changes in the expression and activity of various enzymes. It is likely, therefore, that the factors regulating changes in the expression of genes involved in iron homeostasis at this time are also involved in a host of other more generalized changes.

If this is the case, a greater understanding of the processes involved in intestinal maturation should provide further important clues regarding the regulation of iron metabolism during the neonatal period.

**GRANTS**

This work was supported by a Program Grant from the National Health and Medical Research Council of Australia to G. J. Anderson. D. M. Frazer is supported by a Bushell Postdoctoral Fellowship from the Gastroenterological Society of Australia.

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