Fetal iron status regulates maternal iron metabolism during pregnancy in the rat

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Gambling L, Czopek A, Andersen HS, Holtrop G, Srai SKS, Krejpcio Z, McArdle HJ. Fetal iron status regulates maternal iron metabolism during pregnancy in the rat. Am J Physiol Regul Integr Comp Physiol 296: R1063–R1070, 2009. First published January 29, 2009; doi:10.1152/ajpregu.90793.2008.—Iron metabolism during pregnancy is biased toward maintaining the fetal supply, even at the cost of anemia in the mother. The mechanisms regulating this are not well understood. Here, we examine iron deficiency and supplementation on the hierarchy of iron supply and the gene expression of proteins that regulate iron metabolism in the rat. Dams were fed iron-deficient diets for 4 wk, mated, and either continued on the deficient diet or an iron-supplemented diet during either the first half or the second half of their pregnancy. A control group was maintained on normal iron throughout. They were killed at 0.5, 12.5, or 21.5 days of gestation, and tissues and blood samples were collected. Deficiency and supplementation had differential effects on maternal and fetal hematocrit and liver iron levels. From early in pregnancy, a hierarchy of iron supply is established benefiting the fetus to the detriment of the mother. Transferrin receptor, transferrin receptor 2, and hepcidin mRNA expression were regulated by both iron deficiency and supplementation. Expression patterns showed both organ and supplementation protocol dependence. Further analysis indicated that iron levels in the fetal, and not maternal, liver regulate the expression of liver transferrin receptor and hepcidin expression in the mother.

placenta; deficiency; supplementation

IRON IS AN ESSENTIAL MICRONUTRIENT, required for a wide variety of biological processes. Apart from its obvious role in hemoglobin, it is also central to the normal functioning of many enzyme and catalytic pathways, especially those involved in redox processes. As such, therefore, it is not surprising that iron deficiency leads to a complex series of sequelae (5). We have been studying the consequences of maternal iron deficiency during pregnancy on outcome and on the long-term consequences for the offspring and have shown that deficiency causes low birth weight and hypertension in the offspring (12).

Interestingly, the effects of deficiency early in pregnancy appear to be more severe than those arising when the deficiency occurs later (9). This is surprising, given that most iron is transferred to the developing fetus in the latter part of gestation (13, 16). The data would suggest that the relatively small amounts transferred in the first half are more important for growth and development, while the latter fraction is more important for supplying iron during neonatal life (2, 8, 14).

Whether this is the case in all mammals is not certain. In pigs, for example, much of the iron delivery early in gestation comes from uteroferlin, secreted by the maternal endometrium rather than from serum transferrin (22). In other species, such as cats, the placenta is hemochogasous, and obtains iron from digested maternal red cells (4, 27). In general, however, for those species with a hemochorial placenta, which includes humans, rats, and rabbits, uptake is from transferrin and follows the ontogenic pattern described here and elsewhere (15).

It is clear that the delivery of iron to the fetus, across the placenta or even before the placenta develops, is strictly controlled. There is a hierarchy, such that the levels of iron in the mother can be reduced to 30% of control in iron-deficient animals, while those in the fetus remain up to about 70% of controls (11). To try and understand the mechanisms underlying this regulation, we carried out a series of studies altering maternal iron status midway through pregnancy. We measured iron levels in the maternal and fetal tissues and correlated the data to the expression of the proteins involved in the regulation of iron transfer and storage. We have concentrated on the protein(s) involved in iron uptake (transferrin receptor and transferrin receptor 2) and storage (ferritin H and L) and on the signaling molecules HFE and hepcidin. We have also tested how expression of ferroportin, the iron efflux protein, is altered by the dietary conditions.

Our data demonstrate that the fetal liver plays a central role in regulating iron supply to the developing fetus and further suggest mechanisms whereby this process may operate.

MATERIAL AND METHODS

Experimental diets. The experimental diets were based on a dried egg albumin diet and conformed to American Institute of Nutrition guidelines for laboratory animals (26). FeSO4 was added to achieve levels of added iron of 50 mg/kg (control diet), 7.5 mg/kg (iron-deficient diet), or 75 mg/kg (iron-supplemented diet). Dietary ingredients were purchased from Mayjex (Chalfont-St. Peter, UK), BDH Chemicals (Poole, UK) or Sigma (Poole, UK). Diet formulations have all previously been used (12).

Experimental animals. All experimental procedures were approved and conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. The experiment was performed using 104 weaning virgin female rats of the Rowett Hooded Lister strain. Animals were housed in cages under constant conditions (temperature, humidity, 12:12-h light-dark photoperiod). All of the animals were fed control diet for 2 wk, before being split into two groups. The
first group, 32 animals (group 1), remained on the control diet throughout the experiment, including pregnancy, while the remaining 72 animals were fed an iron-deficient diet for 4 wk before mating with males of the same strain. Mating was confirmed by detection of a vaginal plug, and this day was denoted as day 0.5.

At day 0.5 of gestation, a group of eight rats from both dietary groups were killed and samples were taken (see below). Also at 0.5 days gestation, a group of 24 rats (group 2) were taken off the deficient diet group and given a supplemented diet. At 12.5 days gestation, a group of eight rats from all three dietary groups were killed, and samples were taken (see below). At this point in gestation, the remaining 16 dams in group 2 were placed back on the iron-deficient diet. Also at 12.5 days of gestation, a further 16 rats (group 3) were taken from the deficient diet group and given supplemented diet. The remaining 16 rats (group 4) remained on an iron-deficient diet until the end of experiment. At day 21.5 of gestation, all remaining dams were killed. To summarize, group 1 was a control group (shown as "control" in the figures), group 2 was supplemented in the first half of pregnancy and then returned to a deficient diet (Suppl First). Group 3 was on a deficient diet for the first half of pregnancy and then placed on a supplemented diet (Suppl Second), while group 4 was on a deficient diet throughout pregnancy (Deficient). There was an 80% pregnancy rate in all dietary groups, and no significant difference in litter number between the groups (mean 12.25 ± 0.5). Pups from the dams given iron-deficient diets were smaller than controls (control 4.8 ± 0.06 g, Suppl first 4.0 ± 0.07 g, suppl second 4.5 ± 0.06, deficient 4.1 ± 0.07 g, P < 0.01), as previously described.

On days 0.5, 12.5, and 21.5 of gestation, dams from each appropriate group were anesthetized with isoflurane, and maternal blood tubes (dams) or from the neck (fetuses), which were then killed by cervical dislocation. Livers were dissected from all dams, removed, and immediately frozen in liquid nitrogen before being stored at −80°C. At day 21.5 of gestation, the uterus was removed prior to the liver dissection. Fetuses were delivered by caesarean section and killed by decapitation. The number of fetuses and placentas was recorded. Placentas and livers associated with healthy fetuses were rapidly dissected, weighed, and frozen in liquid nitrogen before being stored at −80°C.

Hematological measurements. Maternal and neonatal hematocrit was measured by drawing blood into capillary tubes from either the blood tubes (dams) or from the neck (fetuses), which were then centrifuged in a high-speed hematocrit centrifuge (Universal 32R, Hettich; Scientific Laboratory Supplies, Coatbridge, U.K.) and read in a microhematocrit reader.

Atomic absorption spectrophotometric analyses. For determination of total iron content in tissues, samples were dried at 90°C under air for 16 h, followed by ashing at 80°C. Hemoglobin was determined by the method of Drabkin (13), and iron was determined by atomic absorption spectrophotometry (Analyst 600, Perkin Elmer, Beaconsfield, UK). Standards and quality controls were included as appropriate. To differentiate between heme and non-heme Fe, samples were precipitated with 20% trichloroacetic acid, as previously described (10).

Real-time quantitative PCR. RNA preparation and real-time quantitative PCR were carried out as previously described (9). Briefly, total RNA from one liver was prepared by use of TRI reagent (Helena Biosciences, Sunderland, UK) and treated with ribonuclease (RNase)-free DNase 1 amplification grade (Invitrogen, Paisley, UK). RNA concentrations were estimated by Agilent analysis (Agilent 2100 Bioanalyzer, Agilent Technologies, Cheshire, UK). First-strand complementary DNA was synthesized by priming with hexamers using the Taqman RT Reagent Kit (Applied Biosystems, Cheshire, UK). Real-time PCR amplification and analysis were performed using a 7700 Sequence Detection System (Applied Biosystems) and ABI prism software version 1.9 (Applied Biosystems). Standard curves were generated from increasing the amount of cDNA made from maternal liver control RNA. The cycle threshold (Ct) values were used to calculate and plot a linear regression line by plotting the logarithm of template concentration (x-axis) against the Ct value (y-axis). These regression lines were used to calculate the expression level (nanogram of total RNA) for unknown samples.

Primers for real-time RT-PCR. Complementary DNA PCR primers for the transferrin receptor 2 (TfR2), HFE, and hepcidin were designed using Primer Express software (ver. 1.5; Applied Biosystems) from the DNA sequences GenBank accession nos. XM_222022, AJ001517 and NM_053469.1, respectively. The primers were as follows: TfR2 forward primer 575 to 596 bp (5′-TGGTCCAGAG- CATCTCGTAA-3′), reverse primer 630 to 651 bp (5′-AAGTCC- CCACATAGTGCGTGTCA-3′), HFE forward primer 581 to 601 bp (5′-TGGGCAAGATCCTTGAATT-3′), reverse primer 661 to 681 bp (5′-GGATCCTGTGCTCTTCCCACT-3′), hepcidin forward primer 201 to 222 bp (5′-AGACACCACTTCCCCCATATGC-3′), and reverse primer 244 to 267 bp (5′-ACAGAGACACAGGGAATTCTT-3′). The primer sets had a calculated annealing temperature of 58°C. Primers were ordered from MWG Biotech, Ebersburg, Germany. To confirm amplification specificity, the PCR products from each primer pair were subjected to gel electrophoreses. For all primer pairs only, one product of correct size was detected (data not shown). The primers for TfR, Ferritin H, and Ferritin L have previously been described (9).

Ferroportin measurement. Total placental homogenate (40 μg) was solubilized in sample loading buffer and subjected to SDS-PAGE. Following immobilization on nitrocellulose, the proteins were exposed to a commercially available anti-ferroportin antibody (Alpha Diagnostics, San Antonio, TX). Cross-reactivity was observed using an horseradish peroxidase-linked secondary antibody (Dako, Cambridge, UK) and ECL Plus (GE Healthcare, Buckinghamshire, UK). Band densities were semiquantified using Scion Image software (Scion, Frederick, MD). At the end of the experiment, the nitrocellulose membranes were stripped (Western Stripping Buffer, Perbio Scientific).
Science, Northumberland, UK) and reprobed with antibodies to actin (Sigma-Aldrich), which acted as loading controls.

Statistical analyses. For each dam, measurements on the fetuses in the litter were averaged, and the data were recorded as a single point for each time of gestation. Data were analyzed by one-way ANOVA with dietary treatments being compared with a post hoc t-test. Linear regression was used to describe linear relationships between continuous variables. Nonlinear relationships were investigated by means of linear regression on log-transformed variables. For some of the relationships between maternal liver TfR and maternal hepcidin, this approach gave unsatisfactory results, and instead, a broken stick model (18), describing a partly linear and partly constant relationship between two variables, was fitted. Mean residual sums of squares from the broken stick and simple linear models were compared with Akaike’s Information Criterion, and if the former were substantially smaller, the broken stick model was preferred. For all regressions, residual plots were inspected for goodness-of-fit, and the result was only deemed acceptable if residuals appeared to be random with approximately constant variance. Significance was assumed at $P \leq 0.05$. The results are presented as means ± SE.

Fig. 2. The effect of maternal iron deficiency and supplementation on maternal liver iron levels during gestation. Results are expressed as the means ± SE of livers from at least six dams.

Fig. 3. The effect of maternal iron deficiency and supplementation on fetal iron status. A: hematocrit. B: liver iron content. Results are expressed as the means ± SE of at least six fetuses, each taken from a different litter. a,b Bars with different letter superscripts are significantly different ($P < 0.05$) by one-way ANOVA.

Fig. 4. Distribution of iron between total and nonheme fractions. Maternal liver (A), placenta (B), and fetal liver (C). Levels are not different (maternal and fetal liver) or significant at $P < 0.001$ (*** Student’s t-test) (placenta). Solid bars denote total iron fraction, Stippled bars denote nonheme fraction. Results are expressed as the means ± SE of samples taken from at least six dams.
Iron-deficient diet for more than 5 wk (Fig. 1) (Hct) up to 12.5 days into pregnancy, despite being on the
at least six dams. Bars with different superscripts are significantly different

Results

Iron status. The dams were able to maintain their hematocrit (Hct) up to 12.5 days into pregnancy, despite being on the iron-deficient diet for more than 5 wk (Fig. 1A). During the second half of pregnancy, iron demands were such that they could not maintain Hct, when measured at day 21 of gestation, even if they were provided with supplementary iron during the first 12.5 days (Fig. 1B). In contrast, supplementation in the second half of pregnancy had restored maternal Hct by the end of pregnancy (Fig. 1B).

Maternal liver iron levels were much more responsive to dietary iron intake compared with maternal Hct. Maternal liver iron levels were already lower in animals on the iron-deficient diets by the beginning of pregnancy (P < 0.05, Fig. 2). This difference increased throughout gestation. Supplementation during the first half of pregnancy was only able to maintain liver iron levels and did not restore values to control levels (P < 0.05, Fig. 2). A similar picture was seen at the end of gestation, with both supplementation groups having improved maternal liver iron levels compared with the deficient group but still significantly less than controls (Fig. 2).

In the fetuses at day 21.5 of gestation, the results for both Hct (Fig. 3A) and liver iron levels (Fig. 3B) mirrored the maternal Hct at the same point in gestation. Supplementation in the first half of pregnancy failed to restore either Hct or liver iron levels to that of control. In contrast, both measures were restored by supplementation during the second half of pregnancy. The iron content of the placenta at day 21.5 of gestation was unchanged by any dietary intervention (data not shown).

We determined whether the patterns of changes of iron were the same for the heme and nonheme fractions by using TCA precipitation. As may be seen (Fig. 4, A and C), there was no significant difference between the two fractions for either the maternal or the fetal livers. We have plotted the correlation data against total iron content of the different tissues. In contrast, in the placenta, nonheme iron was significantly lower, as a consequence of the high blood content. However, the patterns of iron seen under the different dietary conditions were the same for both fractions (Fig. 4B).

Genes of iron metabolism. The effect of maternal iron deficiency and supplementation on the genes involved in regulation of iron metabolism in maternal liver, placenta, and fetal liver was also studied. In the interests of clarity, data are shown only when there are significant changes.

In the maternal liver, mRNA expression of TIR at day 21.5 of gestation were significantly elevated in the iron-deficient dams (P < 0.001, Fig. 5A). Supplementation in either the first or second half of pregnancy decreased expression to that seen in control dams (Fig. 5A). This occurred despite the fact that iron levels in the maternal liver had not returned to normal (Fig. 2). Expression of hepcidin mRNA was decreased in the livers of dams fed iron-deficient diets (P < 0.001, Fig. 5B). Hepcidin expression in the maternal liver at day 21.5 of gestation was restored to control levels by supplementation in the second, but not the first, half of pregnancy (Fig. 5B). None of the dietary interventions altered mRNA expression of TIR2, HFE, and Ferritin H or L in the maternal liver (data not shown).

In the placenta, TIR mRNA expression at day 21.5 of gestation was increased in deficient animals and in those supplemented for the first half of pregnancy. These changes were in the opposite direction to the changes in Hct levels in both maternal and fetal blood and, importantly, to the changes in liver iron levels in the fetus but not the dam. Levels in dams supplemented during the second half had returned to those of

RESULTS

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control (Fig. 6). Placental expression of TfR2, hepcidin, HFE and ferritin H and L mRNA were not significantly altered by dietary treatment. Ferroportin protein levels also did not change in any of the treatments (data not shown).

Unlike the maternal liver and placenta, TfR mRNA expression in the fetal liver was unchanged by maternal iron deficiency or supplementation (data not shown). In contrast, iron deficiency throughout pregnancy resulted in decreased expression of mRNA for TfR2, while both supplementation protocols restored TfR2 expression to control levels (Fig. 7A). Consistent with the pattern seen in maternal liver, expression of hepcidin mRNA was decreased in the livers of fetuses from dams fed iron-deficient diets ($P < 0.001$, Fig. 7B). Supplementation in the second, but not the first, half of pregnancy restored expression of hepcidin in the fetal liver at the end of gestation (Fig. 7B). As with both maternal liver and placenta, none of the dietary interventions altered mRNA expression of HFE, ferritin H, or ferritin L in the fetal liver (data not shown).

Regulation of the genes of iron metabolism. The data generated in these experiments can be used to deduce some of the regulatory pathways involved. Although there was a linear relationship between maternal liver iron levels and maternal liver TfR expression ($R^2 = 0.46$, $P < 0.001$), there is a much stronger correlation between fetal liver iron levels and maternal liver TfR levels ($R^2 = 0.65$, $P < 0.001$). The strongest relationship, and the physiologically most plausible, is between fetal iron levels and maternal TfR when described by a "broken
fetal liver iron (Fig. 11,ations between fetal liver iron and hepcidin expression in the liver iron (data not shown). Instead, there was a linear rela-
ship between placental hepcidin mRNA expression and fetal liver iron when log-transformed (data not shown). In contrast, there is a clear relationship
maternal hepcidin did show a significant correlation with fetal liver hepcidin expression, however, the latter was no longer significant, suggesting that placental TIR mRNA levels are driven by fetal liver iron and not hepcidin.

DISCUSSION

In this paper, we have ascertained the order in which iron stores are maintained during pregnancy and have begun to elucidate some of the mechanisms of regulation. Many of these interactions are inferred by correlation analysis, but the data are clear, and so we believe our conclusions to be sound.

Even at day 0 of pregnancy, we can identify a clear hierarchy in the metabolism of iron. The dam maintains Hct at the expense of reducing iron stores in the liver and can continue to do so until approximately half way through pregnancy. After this, however, the fetal demands are too high (16), and the dam reduces her Hct to maintain fetal supply. The priority of the fetus for iron supply is made most apparent when providing iron during the latter half of pregnancy. Despite the fact that the maternal stores are greatly reduced, iron is delivered to the fetus first, such that its levels are back to control by day 21.5 and the mother’s are still low. Only once the fetal levels are restored do the maternal stores begin to replenish. The data imply, but do not demonstrate directly, that maternal Hct is restored prior to the maternal liver stores. This is, of course, of particular importance in human pregnancy. It suggests that it is very difficult for a pregnant woman to maintain her stores, as has been shown by other authors (reviewed in Refs. 1 and 6), but it also underlies the importance of providing iron supplements (25). At the very least, the supplements will protect the fetus against anemia at birth (21, 23).

The data indicate a clear link between both maternal and fetal liver iron levels and maternal TIR levels. The relationship between fetal iron levels and maternal TIR is much stronger than that described for maternal iron, and including both fetal and maternal iron levels only improved the percentage of the variation explained by ~4%. Thus, we would hypothesize that the influence of fetal liver iron on maternal TIR expression is the most important physiological relationship. The question then arises as to which is the primary cause. Do maternal liver TIR levels drive the fetal liver content, or does the fetal liver regulate iron uptake by the maternal liver? Our data would argue for the latter case. When the dams are given iron supplements early in gestation, the majority of the iron is delivered to the deficient fetus, and maternal levels are not restored until the fetus is back to normal. This is further substantiated by examining the relative levels of TIR in ani-

![Image](http://ajpregu.physiology.org/)

**Fig. 11.** Relationship between fetal liver hepcidin mRNA expression and fetal liver iron levels. Mean liver iron levels were measured and data were plotted against mean liver iron hepcidin mRNA expression at D 21.5 gestation. Statistical analysis was carried out by linear regression. The slope of the line is significantly different from 0, and the data are correlated at P < 0.001, $R^2 = 0.47$. The dashed lines represent the 95% confidence limits.

![Image](http://ajpregu.physiology.org/)

**Fig. 12.** A summary of the proposed model for fetal regulation of iron metabolism during pregnancy. The model shows the pathways identified in these experiments and suggests how the different organs and systems interact. The figure is explained more fully in the DISCUSSION.
mals supplemented in the second half of pregnancy. TfR levels in the maternal liver are at control levels, despite the fact that maternal liver iron is still significantly reduced. In contrast, fetal liver levels are normal. The curve fit showing the relationship between the two parameters is known as a “broken stick” model (18). There is a clear inflection at around 816 μg/g dry weight liver (equivalent to ~1 mg iron per fetal liver at day 21 gestation). We would suggest that above this level constitutes “normal” or “adequate” stores, while below it, the liver activates recovery pathways to reduce the deficiency.

Surprisingly, we could detect no relationship between maternal liver iron and levels of maternal hepcidin expression. There are many studies that show that liver iron regulates hepcidin production and hence iron absorption across the gut (3, 19, 24). However, in this case, it is clearly not so. Rather, the relationship seems to be with the fetal liver. Furthermore, it seems that the gene is either switched on or switched off, depending on the levels of iron in the diet at the time of death. This switch appears to be set to a level of about 1,200 μg/g dry wt in the fetal liver. Curiously, this is very close to the mean value for the control fetal livers, which suggests that if the iron levels in the fetal liver rise above the optimal level, maternal hepcidin is turned on, reducing supply. Clearly, this hypothesis requires further examination.

As would be expected, the placental TfR levels appear to be regulated by fetal liver iron, and the mediator would seem to be hepcidin produced by the fetal liver. How the fetal hepcidin regulates placental TfR is not clear. In other systems, this seems to operate through modulating levels of ferroportin (17). At least at the protein level, this does not seem to be the case in the placenta. However, changes in subcellular localization, rather than increased proteolysis, cannot as yet be excluded.

The question also arises as to how the fetus regulates the maternal iron requirement. There is no relationship between maternal iron levels and placental hepcidin mRNA expression, which suggests, circumstantially at least, that placental hepcidin is not the signaling molecule. There are no data on whether hepcidin can cross from the fetal liver to the maternal circulation, but it would be considered unlikely. We must conclude, therefore, that there are other signaling molecules that remain to be determined.

We can bring together the data in this paper in the diagram (Fig. 12). Briefly, hepcidin from the fetal liver regulates expression of TfR in the placenta. This will modulate iron uptake from the maternal blood. This may act as a signal to the maternal liver, but we hypothesize there will be other, unidentified, signals that regulate iron levels, hepcidin, and TfR in the maternal liver. Placental hepcidin is not this signal.

We also have to consider whether the maternal hepcidin levels follow fetal signals or are simply reflecting the dietary situation. It is difficult to see how the latter can be driving the measured levels. Maternal stores are not related to maternal hepcidin expression. In contrast, we have previously demonstrated that maternal stores do reflect dietary intake in this model (10). The interaction between maternal hepcidin and iron stores in pregnancy clearly merits further investigation, however.

From our data, we can also draw some conclusions about the hierarchy of iron delivery during pregnancy. Iron absorbed from the maternal gut is transferred first to the developing fetus. Within the fetus, the data suggest that the hematocrit is restored before the iron stores. Second, in the queue is the maternal erythron, with maternal stores coming last. Our previous data (9) have shown that maternal iron stores are most closely correlated to fetal growth, with poor iron stores predicting growth reduction. We have also shown that maternal hematocrit and hemoglobin are very inaccurate indicators of iron stores. Our present study suggests, as have others (7, 20), that measuring maternal hemoglobin or hematocrit is not a sensitive or accurate way of determining whether a pregnant woman has sufficient iron to meet her needs and those of her unborn child.

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