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Iron deficiency in the pregnant rat has differential effects on maternal and fetal copper levels

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Abstract

Iron deficiency during pregnancy causes problems both for the mother and fetus. Iron deficiency is known to have secondary effects on copper metabolism. In this study, we use a rat model to examine the effect of iron deficiency on copper levels in maternal and fetal tissue. We assess whether the effects of iron deficiency on copper metabolism are due to alterations in mRNA levels of proteins of copper transport. Rowett Hooded Lister rats were fed diets with four different iron contents before and during pregnancy. Maternal and fetal samples were collected on day 21 of gestation. Copper and iron levels of liver and placenta were analyzed, mRNA levels of genes involved in copper transport were studied, and copper oxidase activity measured. Reduced dietary iron was found to increase maternal liver copper, inversely correlating with iron levels. Correspondingly, copper and ceruloplasmin increased in maternal serum. The placenta showed the greatest increase in copper levels. As the iron content of the maternal diet decreased so did the iron and copper levels in the fetal liver. In all tissues examined, mRNA expression for CTR1, ATOX1, ATP7A, and ATP7B was unchanged by iron deficiency. However, copper oxidase activity in maternal serum and placenta was increased. Our study in a rat model demonstrates that iron deficiency during pregnancy has a differential effect on copper metabolism in the mother and fetus. It is clear from this study that the changes in copper levels that accompany iron deficiency are not mediated by changes in transcription of the genes involved in copper transport. © 2004 Elsevier Inc. All rights reserved.

Keywords: Iron; Copper; Interaction; Pregnancy

1. Introduction

Since the early part of the last century, it has been known that the metabolism of iron (Fe) and copper (Cu) are linked. The initial observation of a link came with a study showing that although Fe supplementation failed to resolve anemia in rats, administration of Cu in the form of either ashed food or acid extracts of the ashes restored hemoglobin levels [1,2]. Several studies since then have confirmed this relationship. Generally, Fe deficiency results in increased Cu levels in the liver and rises in serum ceruloplasmin (Cp) concentrations [3–6].

Fe deficiency is the single most common nutritional disorder world-wide, affecting all age groups to varying degrees, with pregnant women among the most susceptible [7,8]. It is therefore of considerable importance to characterize the effect that this deficiency has on Cu metabolism and to elucidate the mechanisms behind such an alteration. Little is known about the interaction of these metals in the pregnant animal or her offspring, and few studies have examined the effect of Fe deficiency on Cu metabolism during pregnancy [9,10].

Although it has been clearly established that deficiencies of either Fe or Cu alter the distribution of the other mineral, the mechanisms have not been characterized. In the last few years the steps involved in cellular Cu transport have begun to be understood. Uptake is through a carrier-mediated process, probably involving the Cu transporter Ctr1p [11]. After uptake, Cu binds to Atox1, one of a series of Cu chaperones [12]. Atox1 carries the Cu to either Atp7A or Atp7B, depending on the tissue [13,14]. In the liver, Atp7B supplies Cu for Cp synthesis [15]. In the placenta, Atp7A is central to Cu efflux [16,17]. Whether the changes in Cu status generated by Fe deficiency alter expression of these genes is not known.

We have developed a model for mild maternal Fe deficiency to examine changes in Fe metabolism, growth, and development. The dietary regimen applied causes mild but

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significant maternal anemia, associated with reduced Fe levels in both maternal and fetal tissue. The reduction in maternal dietary Fe also leads to a significant reduction in fetal weight and an up-regulation of many of the proteins involved in Fe transport [18,19]. The purpose of our current study, using our established rat model of maternal Fe deficiency, was to gain a better understanding of the relationship between Fe and Cu during pregnancy. Our second objective was to determine whether we could explain any changes in Cu metabolism by altered expression of the genes encoding the proteins of Cu transport.

2. Methods and materials

2.1. Experimental animals and diets

Experiments were performed using weanling female rats of the Rowett Hooded Lister strain. A total of 40 female weanling rats were fed control diet for 2 weeks, before being randomized into four groups. The first group of rats (16 animals) remained on control diet (50 mg Fe kg^{-1} diet), whereas the remaining three groups (8 animals each) were placed on experimental diets of reduced Fe content (37.5, 12.5 and 7.5 mg Fe kg^{-1} diets). All diets were freely available and body weights were recorded three times per week throughout the experiment. All groups were fed these diets for 4 weeks before mating. The rats were mated with males of the same strain. The female rats were maintained on the same diet throughout pregnancy and killed at day 21 of gestation. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

The experimental diets used were based on a dried egg albumin diet [19,20] and conformed to American Institute of Nutrition guidelines for laboratory animals [21]. $FeSO_4$ was added to achieve total levels of Fe of 50 (control diet), 37.5, 12.5, and 7.5 mg $\cdot$ kg^{-1} of diet. Diets were identical in all other respects. Dietary ingredients were purchased from Mayjex Ltd. (Chalfont-St Peter, UK), BDH Chemicals, or Sigma (Poole, UK).

2.2. Tissue samples

Placentas associated with healthy fetuses were weighed and frozen in liquid nitrogen before being stored at −70°C. Livers were dissected from all dams and from eight fetuses, which were chosen from each dam at random, removed, and immediately frozen in liquid nitrogen before being stored at −70°C. Maternal and fetal blood were collected in nonheparinized tubes, centrifuged at 1000 $\times$ *g*, at 4°C for 10 minutes. The resulting serum was stored in metal-free Ependorf tubes at −70°C.

2.3. Atomic absorption spectrophotometric analysis

The Cu and Fe content of tissue and serum samples were determined by graphite furnace atomic spectrophotometry (model 3100, Perkin-Elmer Corp., Norwalk, CT), according to standard procedures, as previously described [19]. Standard curves for Cu and Fe was prepared from commercially available standards (Spectrosol BDH, Poole, UK). Appropriate quality controls were included as necessary.

2.4. Cu oxidase analysis

Serum Cp oxidase activity was assayed by measuring the rate of oxidation of ρ-phenylenediamine according to the protocol of Sunderman and Nomoto [22]. This method was adapted, as previously detailed [23], to measure placental Cu oxidase activity. Cp oxidase and Cu oxidase activity was calculated as units $\cdot$ mL^{-1} serum and units $\cdot$ mg^{-1} protein respectively, where unit is $\Delta mA \cdot h^{-1}$.

2.5. Northern blot analysis and cDNA probes

RNA was extracted from tissue using Tri reagent (Genetic Research Instrumentation, Baintree, UK) and analyzed by Northern blotting as previously described [18]. The mRNAs were quantified by measuring the amount of radioactivity hybridizing to the bands on the Northern blot and corrected for loading by reprobing with a probe for 18S rRNA. The data were expressed as disintegration per minute and are the mean $\pm$ SEM of *n* values. ATP7A, ATP7B, and CTR1 probes (576, 844, and 452 bp respectively) were prepared by reverse transcriptase–polymerase chain reaction from rat placenta RNA using standard protocols (Promega, Southampton, UK) and primers designed from published sequences. The sense and antisense primers, respectively corresponded to bases 3865-3884 and 4440-4421 of rat ATP7A (GenBank accession no. U59245), 621-640 and 1465-1446 of rat ATP7B (GenBank accession no. NM_012511), and 305-326 and 729-710 of rat copper transporter 1 (CTR1) (GenBank accession no. AF268030). Cp and ATOX1 specific probes were obtained from the UK HGMP Resource Centre, I.M.A.G.E. consortium Clone ID 0522108 and 1328907, respectively [24]. The cDNA probe was labeled with (α-^{32}P)dCTP with a Megaprime labeling kit (Amersham, Buckinghamshire, UK). Specificity of all probes was determined by sequencing before being used for Northern blot analysis.

2.6. Statistical analysis

Samples for each analysis were taken from each dam and from a fetus chosen at random from each litter. If more than one fetus was sampled from any one litter, the data are averaged and used as a single data point. This is the statistically more accurate option, although it may obscure trends that might be identified with high numbers of animals.

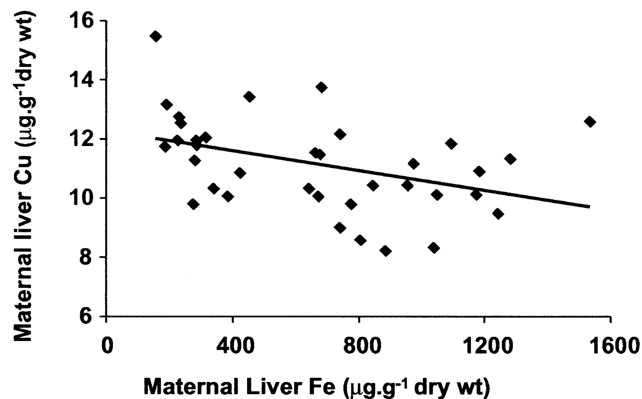

Fig. 1. Maternal Fe deficiency induces an increase in maternal liver Cu. Samples were taken from maternal liver at day 21 of gestation and were treated as described in the Methods and materials section before being analyzed by atomic absorption spectroscopy. Each individual dam is represented as a single data point. Line represents calculated linear regression, $P = 0.01$.

Where appropriate, regression analysis and one-way analysis of variance were used to compare multiple data sets, whereas the Student t test was used to compare two data sets. Correlation coefficients (R) are presented for all regression analyses. Significance was assumed at $P < 0.05$. Analysis was performed using Excel 6.0 (Microsoft Corp., Seattle, WA).

3. Results

There were two ways of displaying the relationship between Fe status and Cu levels, in relation to either maternal dietary intake or maternal tissue Fe levels. The latter is clearly the more accurate method and, wherever possible, we have presented our data using this format.

Cu levels in the maternal liver are inversely correlated to maternal liver Fe levels ($P = 0.01$, $R = 0.40$) (Fig. 1). At the same time, serum Cp ($P = 0.02$, $R = 0.41$) (Fig. 2A) and Cu levels increase proportionately to decreasing maternal liver Fe ($P = 0.02$, $R = 0.39$) (Fig. 2B). In contrast, mRNA levels for the proteins involved in cellular Cu metabolism in the liver (CTR1, ATOX1, ATP7B, and Cp) are not changed (Table 1).

In the placenta, Cu levels are also inversely related to placental nonhemetologic Fe content ($P = 0.001$, $R = 0.59$) (Fig. 3A). Interestingly, there is also a strong relationship between maternal liver Fe and placental Cu ($P = 0.03$, $R = 0.37$, Fig. 3B). As in the liver, CTR1, ATOX1, and ATP7A mRNA levels are not changed by either the Fe deficiency or the associated Cu increase (Table 2). Fe efflux from the placenta is mediated at least in part by a Cu oxidase [23,25], and the activity of this enzyme increases as maternal liver Fe levels decrease ($P \leq 0.001$, $R = 0.64$) (Fig. 4). Although the activity of the placental Cu oxidase shows a strong relationship with the maternal Fe status, it shows no significant correlation with either placental or conceptus (*i.e.*, placental plus fetal) Fe levels (data not shown).

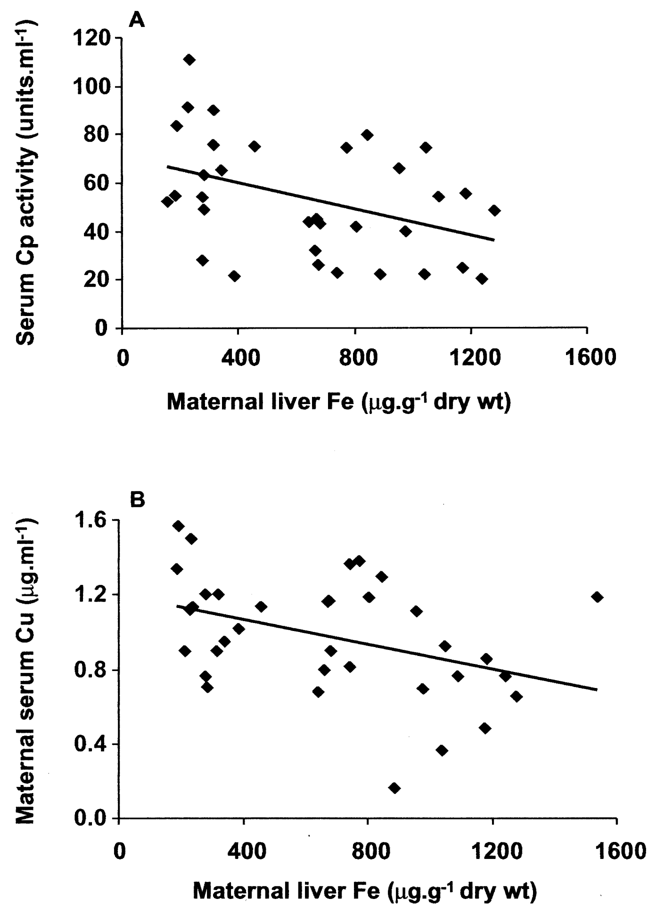

Fig. 2. Maternal Fe deficiency induces an increase in maternal serum Cp activity (*A*) and Cu (*B*) levels. Samples were taken at day 21 of gestation and treated as described in the Methods and materials section. Each individual dam is represented as a single data point. Lines represent calculated linear regressions, $P = 0.02$.

In the fetus, the pattern is different. As Fe levels in the maternal diet decrease, so do fetal liver Cu levels ($P =$

Table 1
Effect of maternal Fe deficiency on expression of mRNA for proteins involved in copper metabolism in maternal liver

	Control ($50\ mg \cdot kg^{-1}$)	Deficient ($7.5\ mg \cdot kg^{-1}$)	*N*
CTR1			
5.5 kb	1952 ± 80	1785 ± 108	12
2 kb	902 ± 71	868 ± 114	12
ATOX1	3460 ± 349	3757 ± 346	6
ATP7B	2274 ± 146	2499 ± 179	12
Cp	32448 ± 4834	37150 ± 7386	12

Maternal liver mRNA was isolated, separated, and hybridized as described in the Methods and materials section. The data were normalized to 18S RNA, expressed as disintegration per minute, and are the mean ± SEM of *n* values. There were no significant differences between control and deficient samples.

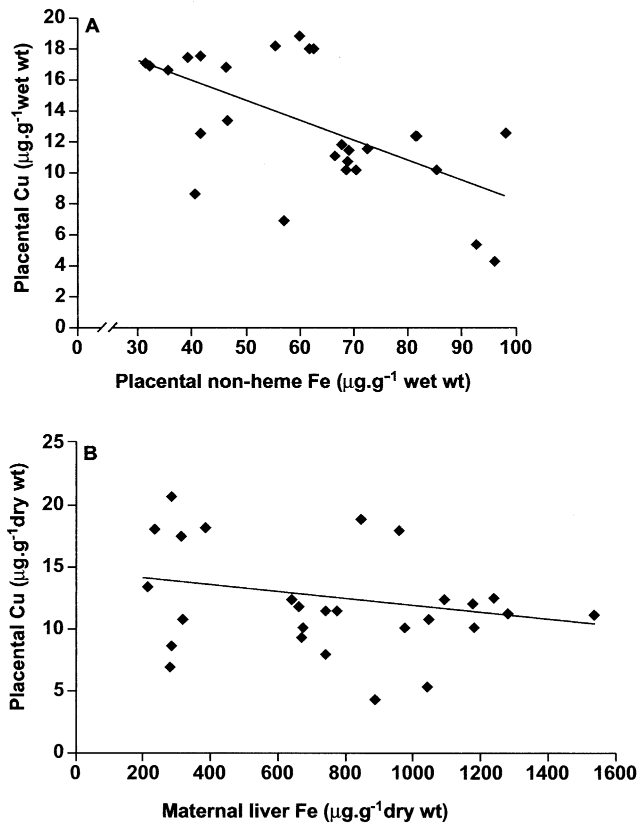

Fig. 3. Placental Cu levels are inversely proportional to placental non-hemetologic Fe (*A*) and maternal liver Fe (*B*). Placental samples were treated as described in the Methods and materials section before being analyzed by atomic absorption spectroscopy. Each individual data point represents one placenta from one dam. Lines represent calculated linear regressions, $P = 0.001$ and 0.03 respectively.

0.005) (Fig. 5). However, these changes are not correlated with either maternal or fetal liver Fe levels or with any other fetal Fe level (data not shown). Similarly, Cu and Cp levels in the fetal serum are not altered by Fe status (data not shown). As with the other tissues studied, the mRNA levels of the proteins involved in copper metabolism (CTR1, ATOX1, ATP7B, and Cp) are not changed in the fetal liver (Table 3).

Table 2
Effect of maternal Fe deficiency on expression of mRNA for proteins involved in copper metabolism in placenta

	Control ($50\ mg \cdot kg^{-1}$)	Deficient ($7.5\ mg \cdot kg^{-1}$)	*N*
CTR1			
5.5 kb	691 ± 120	631 ± 100	12
2 kb	418 ± 70	410 ± 32	12
ATOX1	3382 ± 442	3268 ± 273	6
ATP7A	3183 ± 543	3330 ± 435	12

Placental mRNA was isolated, separated, and hybridized as described in the Methods and materials section. The data were normalized to 18S RNA, expressed as disintegration per minute, and are the mean ± SEM of *n* values. There were no significant differences between control and deficient samples.

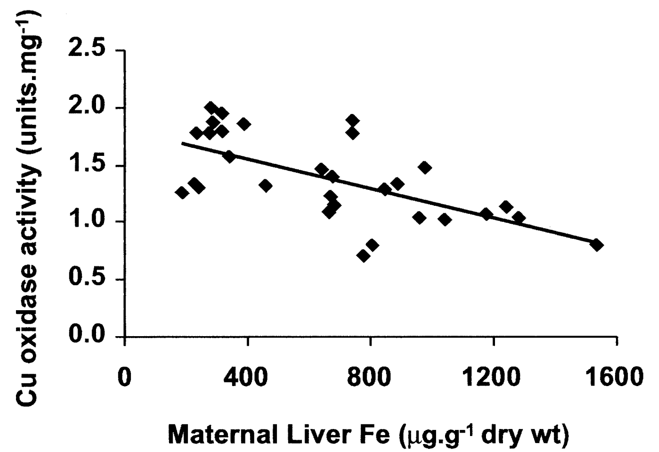

Fig. 4. Placental Cu oxidase activity is increased as iron levels in the placenta decrease. Samples were taken from maternal liver at day 21 of gestation and were treated as described in the Methods and materials. Each data point represents a single placenta from each dam. Line represents the calculated linear regression, $P \leq 0.0001$.

4. Discussion

This study has examined the effect of different degrees of maternal Fe deficiency during pregnancy on Cu levels and expression of proteins of Cu metabolism. This model of mild maternal Fe deficiency causes significant falls in maternal hemoglobin (180 to 150 $g \cdot mL^{-1}$) and serum Fe (1 to 0.3 $\mu g \cdot mL^{-1}$), but total iron binding capacity remains unchanged, suggesting only a mild state of Fe deficiency. This is reflected in a reduction in maternal and fetal liver Fe content and fetal weight, but no difference in fetal number [19]. Interestingly, the effects on Cu metabolism were op-

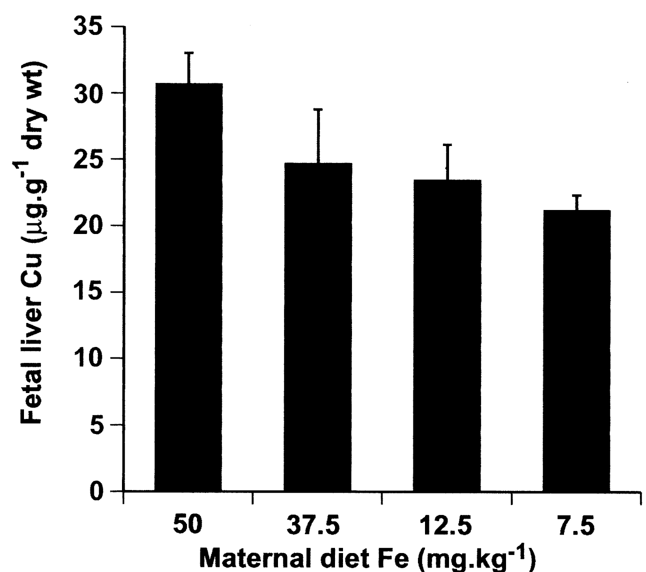

Fig. 5. Fetal liver Cu levels are decreased by dietary Fe deficiency, $P = 0.005$. Fetal livers were collected and treated as described in the Methods and materials section before being analyzed by atomic absorption spectroscopy. Results are mean ± SEM of eight samples in each group.

Table 3
Effect of maternal Fe deficiency on expression of mRNA for proteins involved in copper metabolism in fetal liver

	Control ($50\ mg \cdot kg^{-1}$)	Deficient ($7.5\ mg \cdot kg^{-1}$)	*N*
CTR1			
5.5 kb	761 ± 42	882 ± 100	12
2 kb	364 ± 150	295 ± 105	12
ATOX1	5359 ± 340	4961 ± 403	6
ATP7B	1246 ± 237	1008 ± 140	12
Cp	1815 ± 135	1570 ± 267	12

Fetal liver mRNA was isolated, separated and hybridized as described in the Methods and materials section. The data were normalized to 18S RNA, expressed as disintegration per minute, and are the mean ± SEM of *n* values. There were no significant differences between control and deficient samples.

posite in the mother and fetus: maternal liver Cu content increased, whereas fetal liver decreased.

The increase in maternal liver Cu content during Fe deficiency was not unexpected. Previous studies in nonpregnant animals have shown that Fe deficiency induces an increase in liver Cu levels. The levels vary depending on the study, between 50% and 300% control [3,5,26]. This interaction has been less well studied during pregnancy, however. The only previous detailed study in a rat model showed an unexpected decrease in maternal liver Cu and an increase in maternal serum Cp levels [10]. The authors themselves point out the data as surprising, and their own previous work shows the same relationship between Fe and Cu as that presented here [9]. There are several possible explanations from the differences. The ratio between Cu and Fe in the control diets used in the two studies is markedly different, 1:30 in the Sherman diet [10] and 1:10 in our diet, a ratio similar to that recommended for rodent diets by the American Institute of Nutrition [21,27]. It is also possible that the differences between the two studies relates to the degree of Fe deficiency achieved [19]. A comparison of Fe status indicators suggests that the degree of deficiency achieved in the present study is marginally greater. However, it is difficult to see how this difference, which is not enormous, could result in such a dramatic change in Cu metabolism.

The most likely cause of the differences between the two studies is the length of time spent on the diet. In the first study examining the relationship between liver Cu and Fe levels in the rat, Sourkes et al. showed that a change in hepatic Cu levels was not evident until the rats had been on Fe-deficient diets for 7 weeks [3]. The dietary protocol used in this study resulted in the dams having been on experimental diets for 7 weeks by the end of gestation. Sherman and Moran, however, placed the dams on iron-deficient diets on the first day of pregnancy, allowing only 3 weeks on Fe-deficient diet before the end of gestation [10].

The increase in Cu levels in the placenta is greater than that seen in any other tissue, including the liver. Cu transfer from mother to fetus is mediated by at least one transporter in the microvillar membrane. The placenta has only a limited capacity to cope with maternal Cu deficiency, and reduced maternal Cu leads to a drop in fetal levels [28,29]. In contrast, increasing Cu does not lead to overload, suggesting that the placenta can limit Cu transfer [30,31]. In support of this, the proportion of total (maternal plus fetal) Cu in Fe-deficient fetuses is lower than control. The apparent Km of the transporter is 1-4 μmol/L and is a passive, carrier-mediated process, so Cu uptake would be unlikely to be the rate-limiting step [32,33]. Instead, we hypothesize that the increase in placental Cu results from regulation at the efflux step. This clearly remains to be demonstrated.

This hypothesis may also explain why there is no correlation between fetal Fe and fetal Cu levels. The result contrasts with postnatal, but not prenatal, data produced by Sherman and Moran. Although they showed no change in liver Cu levels in day 21 fetuses [10], an increase in Cu levels was seen the livers of 2-day-old pups born to Fe-deficient dams [9]. We would suggest that the placenta protects against Cu overload and that this protection disappears postnatally.

It has been known for some time that micronutrients can regulate the expression of the proteins involved in their transport and metabolism at the transcriptional as well as translational level [34,35]. Not only do micronutrients regulate the expression of proteins involved in their own metabolism, but they can also regulate expression of proteins involved in that of other metals. For example, it has been demonstrated that selenium deficiency caused an increase in mRNA expression for proteins involved in iron metabolism [36]. Therefore, as a first step toward identifying the pathways, we have examined the regulation of the Cu transport proteins at the transcriptional level.

Cu uptake is a carrier-mediated mechanism, using Cu-His_2 as the substrate but with only the Cu being carried across the membrane [37,38]. We tested the hypothesis that changes in Cu levels as a consequence of Fe deficiency occurred a result of increased expression of CTR1. Ctr1p is the proposed high affinity Cu carrier and is thought to be the rate-limiting step for copper uptake in *Saccharomyces cerevisiae* [39,40]. The data presented here show no change in expression of mRNA in any of the tissues examined. This does not, of course, completely exclude increased uptake through CTR1, inasmuch as Ctr1p is also controlled by post-translational regulation [41].

In terms of the effect of Fe deficiency on Cu metabolism, it is the genes involved in the intracellular trafficking, ATOX1, the copper chaperone, and ATP7A/B, the Cu-ATPases, which are of particular interest, as their yeast homologues fall under the transcriptional control of the iron responsive trans-activator Aft1p [42–44]. There was, however, no significant change in mRNA expression of either the Cu chaperone, ATOX1, or the Cu-ATPases, ATP7B in either the maternal or fetal liver, or of ATP7A in the placenta.

The absence of changes in ATP7A/B mRNA expression was not entirely unexpected. The Wilson and Menkes gene products, ATP7A and ATP7B, are ATPases involved in intracellular translocation and excretion of copper [45]. Under normal conditions, they are primarily found in the trans-Golgi network. In hepatocytes of copper loaded rats, neither mRNA nor protein levels of ATP7B change. However, as Cu concentrations within the cell increased, ATP7B relocated to the plasma membrane [46]. Similar results have been shown for ATP7A in Chinese hamster ovary cells [17]. Together these findings suggest that the Cu-ATPases are subject to post-translational rather than transcriptional regulation by Cu. Such changes would not have been detected in this study; however, the data do rule out the possibility of an Aft1p-type regulation in mammalian cells *in vivo.*

In contrast, levels of the placental oxidase do increase. These results are supported by others from our laboratory in a cell culture model [23]. We have shown that the enzyme is up-regulated by Fe deficiency and also by Cu loading. At present we cannot determine the primary signal for up-regulation in the placenta; however, several pathways can be proposed, as described above.

In summary, this study demonstrates that Fe deficiency during pregnancy has a differential effect on Cu metabolism in the mother and fetus. The interaction between Fe and Cu in the pregnant rat is consistent with that seen in the non-pregnant animal, whereas the opposite effect is seen in the fetus. We have also eliminated some possible explanations for these observations. It is clear from this study that the changes in Cu levels that accompany Fe deficiency are not mediated by changes in transcription of the genes involved in Cu transport. It is apparent, however that further investigations are required to clarify the mechanisms involved in the interaction of Fe and Cu metabolism during pregnancy.

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