Copper deficiency increases iron absorption in the rat

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In contrast to rodents, copper deficiency in swine results in decreased levels of total liver iron, suggesting that iron absorption is impaired (17, 23). In fact, in these animals, the enterocytes accumulate large amounts of ferritin-bound iron (23), suggesting that iron import into enterocytes is normal but export is impaired. Supporting this, direct measurement showed that iron absorption was reduced in swine (23). Irrespective of these differences between the swine and rodent, as plasma iron levels fall, anemia follows (7, 8, 13, 14, 17, 21, 24, 25, 30, 34, 35, 45, 46).

The discovery of the copper-containing plasma protein ceruloplasmin (Cp) and its subsequent characterization as a ferroxidase led to the hypothesis that in hepatocytes, macrophages, and enterocytes, iron is oxidized during efflux to bind to plasma transferrin (19, 29). However, Williams and co-workers (46) showed that the ferroxidase activity of Cp varies widely between species, being lowest in the rat and 10-fold higher in swine, which led the authors to conclude that this action may not be the major function of Cp but that it only enhances the export of iron. Certainly, intact Cp is necessary for the export of iron, because iron accumulated in the liver of individuals with aceruloplasminemia and in Cp knockout mice (18, 47).

In Cp knockout mice, intestinal iron transport was not affected (18, 47) nor was Cp effective in augmenting iron efflux from enterocytes in whole tissue (9) or in cell lines (40, 49), suggesting the functioning of an alternative protein to Cp. Indeed, it was recently shown that for optimal efflux of iron, the enterocyte requires the Cp homolog hephaestin (Hp) (16, 42), and Attieh and co-workers (2) recently showed that it has ferroxidase activity comparable with that of ceruloplasmin. Therefore, it might be expected that if Hp operates by way of its ferroxidase activity in iron absorption, then this will be impaired by copper deficiency to a similar extent as Cp in hepatocytes. The function of Hp in iron absorption is, in part, based on studies in mice with sex-linked anemia (slала), in which three exons of the Hp gene are missing (1, 42). The intestinal phenotype of sla mice is similar in appearance to copper-deficient swine; that is, the export of iron from the intestine is impaired, and there is an accumulation of ferritin-bound iron within the enterocyte (3, 10–12, 22, 31, 32). However, when these mice

THE BODY'S USEABLE IRON pool is determined by the amount absorbed by the intestine plus that released from storage sites such as macrophages and hepatocytes (18). It has been shown that adequate copper stores are necessary for the efflux of iron from the liver. Thus, during copper deficiency in rats, iron accumulated within hepatocytes (7, 8, 13, 14, 24, 25, 30, 33, 45). The finding that liver iron was increased in proportion to dietary iron content suggests that iron absorption may be normal or even increased by copper deficiency. However, direct measurements of iron absorption in copper-deficient rats are inconclusive (5, 7, 9).
were fed an iron-deficient diet for several days during which time a new population of enterocytes with low cellular iron enters the absorptive zone of the villus. Iron absorption increased, and in some studies, this was to levels seen in normal animals fed the same diet (11, 36, 39, 41). This finding suggests that the mutant Hp can function normally during iron deficiency of the enterocyte and raises the question as to whether Hp operates by way of its ferroxidase activity or by another mechanism that is compromised by the mutation.

Because of the impaired iron absorption reported in copper-deficient swine and sla mice, it is expected that copper-deficient rodents will respond similarly, but direct and indirect measurements do not support this (5, 7, 9). Furthermore, it was recently shown that in copper-deficient Caco-2 cells, iron absorption was increased (49), although others (38) have found that in these cells, exposure to copper increased the activity of the basolateral transporter ferroportin1 and cellular efflux of iron. In view of these discrepancies, we studied iron absorption in copper-deficient rats, and these data correlated with the expression of the apical and basolateral iron transporters DMT1 and ferroportin1, respectively, along with mucosal ferritin and nonheme iron, which are important factors known to contribute to the mechanism of iron absorption.

**MATERIAL AND METHODS**

**Animals**

The Animal Welfare Committee of the University of Western Australia has approved all procedures dealing with the handling of animals described in this study. Normal, weanling outbred female Wistar rats were obtained from the Animal Resource Center (Murdock, Western Australia). The rats were placed on one of three diets, each containing identical protein, fat, carbohydrate, complete vitamin, and fiber supplements. The diets were produced in accordance with the recommendations of the American Institute of Nutrition (AIN 93) (43). The diets also contained a balanced mineral mix differing only in the iron and copper contents. The copper-deficient diet contained no added copper but had a normal level of iron (70 mg/kg as finely ground ferric citrate). The control diet contained normal levels of copper (6 mg Cu(II)/kg) and iron (43), and the iron-deficient diet had normal levels of copper but no added iron.

**Iron Absorption**

Preparation of iron absorption test solution. The Fe(II) test dose consisted of a 200-μl solution containing 200 nmol of iron in the form of FeSO₄ to ascorbate at a molar ratio of 1:100 in 50 mM HEPES and 80 mM NaCl. This solution was prepared immediately before use by mixing 59FeSO₄ in 50 mM HCl with 100-fold molar excess of unlabeled FeSO₄ in 50 mM HCl and then the required amounts of ascorbic acid. After it was mixed, NaCl, HEPES (pH 7.0), and NaOH were added in amounts required to give the correct iron, HEPES, and NaCl concentrations and to neutralize the acid in the iron solutions to pH 7.0. The Fe(II) to ascorbate molar ratio of 1:100 was chosen as suitable for maintaining the iron in a soluble, absorbable form in the ferrous state (36).

Animals were lightly anesthetized with Halothane before gavage with Fe(II). Whole carcass radioactivity was then measured immediately and again 5 days later, at which time carcass radioactivity had stabilized. Absorption represented the percentage radioactivity remaining in the carcass divided by the total amount injected.

**Isolation of Villus Enterocytes**

The proximal small intestine was removed, and the enterocytes along the crypt-villus axis were separated into 10 fractions as previously described (28). Fractions 2–4, containing enterocytes derived from the midvillus region, were pooled and washed three times in PBS to remove extracellular mucus. Total cell copper concentration was measured by atomic absorption spectrometry and corrected for variations in cellular protein (40). Total copper-dependent oxidase activity was measured as described previously (40).

**Recovery of Tissue for Analyses**

Five days after gavage, the animals were anesthetized with pentobarbital sodium (Nembutal) at a dose of 12 mg/100 g body wt. After deep anesthesia, a midline abdominal incision was made into the abdomen and then into the thorax. A blood sample was taken from the heart for the measurement of hematocrit and plasma iron. The animal was then exsanguinated. The liver was removed, weighed, and a 200-μg portion of the right lobe was placed in 10% buffered formal saline (BFS). The duodenum was removed, and a 5-mm segment from the proximal end was fixed in BFS. The remaining duodenum was opened longitudinally, and then a superficial scraping of the duodenal mucosa was homogenized in lysis buffer consisting of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl plus 0.5% Triton X-100, and protein inhibitors PMSF, leupeptin, and Trasylol for Western blot analysis and the measurement of nonheme iron. A piece of liver was also prepared for Western blot analysis.

**Plasma ferroxidase I activity**

Plasma ferroxidase I (ceruloplasmin) was measured by incubating plasma with 9.2 mM p-phenylenediamine in 0.2 M acetate buffer pH 5.2 in the presence or absence of 1.5 M sodium azide at 37°C. The reaction product was measured at 540 nm, and the plasma ferroxidase activity was converted to grams per liter expressed as units (29).

**Nonheme Iron Levels in Duodenal Mucosa, Liver and Plasma**

Nonheme iron was measured in plasma, liver and intestinal mucosa (20).

**Expression of DMT1-IRE, ferritin, and ferroportin1 by Western Blot Analysis**

The protein content of samples of the liver and intestinal mucosa was estimated by the biuret method. One hundred micrograms of total cellular protein were electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel as described previously (40). Staining of the gel with Ponceau S was performed to show equal loading of lanes before Western blotting. The blots were immunoreacted with polyclonal antibodies generated against human DMT1-IRE (NRAMP22-S, Alpha Diagnostics International), rat ferroportin1 (40), and rat L&H ferritins (40) and processed for Western blot analysis as described previously (40). The human DMT1-IRE recognizes rat DMT-1 antibody as evidenced by immunodetection of a protein migrating at the same rate from total protein derived from rat IEC-6 and rat enterocytes (unpublished observation). The density of the signal and background
were determined by densitometry using the National Institutes of Health Image 1.62 to estimate the level of expression.

**Detection of Iron and Ferritin/Hemosiderin in the Duodenum and Liver**

Five-micrometer paraffin sections of duodenum and liver were stained for iron by the Perl’s staining method. Staining was performed using 1% potassium ferrocyanide dissolved in either 2% HCl to identify hemosiderin deposits or in 5% acetic acid to detect soluble-labile iron (6).

**Detection of Ferritin in the Duodenum**

Sections were also prepared for immunohistochemistry to detect ferritins (40) as previously reported (27).

**Statistics**

Data were analyzed by either the Student’s t-test for unpaired samples or when three conditions were analyzed collectively by analysis of variance with partitioning according to Tukey’s test. Significance was considered at *P* < 0.05.

**RESULTS**

**Validation of the Model**

There was no significant difference in plasma ferroxidase I activity between control and iron-deficient rats (Table 1). However, plasma ferroxidase I activity was significantly reduced from these levels in copper-deficient rats (Table 1). Copper deficiency of duodenal enterocytes was confirmed because copper concentration was reduced to 32% normal levels (Table 1) and total enterocyte copper-dependent oxidase activity was reduced to 46% normal levels (Table 1).

**Body and Tissue Weights and Hematological Indexes**

Total body and liver weights were not different between the three conditions (Table 1). Plasma iron and hematocrit were significantly reduced from controls in animals made either copper deficient or iron deficient (Table 1).

**Liver and Spleen Nonheme Iron**

Compared with controls, liver nonheme iron was increased with copper deficiency and significantly reduced with iron deficiency (Fig. 1A). Also, because there was no difference in the weight of the livers among the three groups, the same pattern was seen when the iron concentrations were corrected for slight variations in weight (Fig. 1A).

In the spleen, copper deficiency did not alter nonheme iron stores compared with control, but iron deficiency reduced nonheme levels compared with copper deficiency and control animals (Fig. 1B). This was the case when the data were corrected for variations in weight (Fig. 1B).

**Perl’s Staining within the Liver**

Little iron staining was seen in the livers of control animals (Fig. 2A) and none was detected in the livers of iron-deficient animals (data not shown). Liver iron staining was evident in copper-deficient animals, where it was highest within perportal hepatocytes and least in those surrounding the central vein region (Fig. 2B). Kupffer cells appeared negative for iron staining (Fig. 2).

**Iron Absorption**

Total iron absorption was significantly greater in animals made copper deficient compared with control animals. However, in iron deficiency, total iron absorption was greater than in copper-deficient and control animals (Table 1).

**Duodenal Mucosal Nonheme Iron Concentration**

Copper deficiency and iron deficiency significantly reduced mucosal nonheme iron concentration compared with control animals. Mucosal iron levels were significantly lowered in iron deficiency compared with copper deficiency (Table 1).

**Table 1. Plasma ferroxidase I activity, enterocyte copper levels, total enterocyte copper-dependent oxidase activity, body and liver weights, plasma iron, hematocrit, expression of DMT1-IRE, ferritin, and ferroportin1 in 12-wk-old rats fed a copper-deficient, normal, or iron-deficient diet to female rats for 8 wks from weaning**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Copper Deficiency</th>
<th>Controls</th>
<th>Iron Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ferroxidase I, U</td>
<td>12,12,4</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.0A</td>
<td>0.3 ± 0.0A</td>
</tr>
<tr>
<td>Enterocyte copper, fg/μg protein</td>
<td>3.3</td>
<td>95 ± 18</td>
<td>282 ± 59A</td>
<td>NP</td>
</tr>
<tr>
<td>Enterocyte copper-dependent oxidase activity, U/ng protein</td>
<td>3.3</td>
<td>13 ± 3</td>
<td>28 ± 1A</td>
<td>NP</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>24,27,5</td>
<td>188 ± 5</td>
<td>207 ± 5</td>
<td>219 ± 10</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>22,20,5</td>
<td>91.0 ± 0.8</td>
<td>92.0 ± 4</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>Plasma iron, μg/ml</td>
<td>4,4,4</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.1A,C</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>5,5,4</td>
<td>33 ± 1</td>
<td>41 ± 3A,C</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>Iron absorption, % dose injected</td>
<td>2,3,1,5</td>
<td>55 ± 4</td>
<td>44 ± 4A,C</td>
<td>84 ± 5</td>
</tr>
<tr>
<td>Mucosal nonheme iron, ng/μg protein</td>
<td>10,9,5</td>
<td>0.25 ± 0.04</td>
<td>0.46 ± 0.08A,C</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Mucosal DMT1-IRE, AU/100 μg protein</td>
<td>1.2</td>
<td>140</td>
<td>145</td>
<td>NP</td>
</tr>
<tr>
<td>Mucosal ferritin, AU/100 μg protein</td>
<td>4,5,3</td>
<td>88 ± 5</td>
<td>136 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Mucosal ferroportin1, AU/100 μg protein</td>
<td>2,2</td>
<td>125</td>
<td>131</td>
<td>NP</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. Superscripts indicate the group in which a significant difference exists from the designated group. NP, not performed.
Comparisons Among Duodenal Mucosal Nonheme Iron Concentration, Ferritin Expression, and Iron Absorption

When these data from control, copper-deficient, and iron-deficient rats were pooled and correlations were performed between mucosal nonheme iron and ferritin expression (Fig. 3A), mucosal nonheme iron and total iron absorption (Fig. 3B), and ferritin expression and total iron absorption (Fig. 3C), strong correlations were found. The strongest correlation existed between mucosal nonheme iron and ferritin expression, but the other relationships were also significant.

DMT1, Ferroportin1, and Ferritin Expression of Villus mucosa

The levels of DMT1-IRE and ferroportin1 expression were similar in copper-deficient and control conditions. DMT1-IRE and ferroportin1 were detected with molecular weights of ~66 and ~60 kDa, respectively (Fig. 4, A and B). The ferritins were detected as a single band migrating at ~20 kDa (Fig. 4C). Compared with control animals, mucosal ferritin was reduced in animals deficient in copper and, to a greater degree, by iron deficiency (Table 1, Fig. 4).

Staining of Duodenum for Iron and Cellular Distribution of Ferritin

There was no detectable staining by the Perl's method in the duodenum of any condition studied (data not shown). This was the case when the reaction was carried out to reveal soluble iron using 5% acetic acid or stored iron in the form of hemosiderin using 2% hydrochloric acid (data not shown).

In control animals, ferritin expression commenced at the crypt-villus junction within enterocytes and reached maximal intensity at the villus tip (Data not shown). In copper-deficient animals, ferritin expression was less than in control animals. It was largely confined to the upper one-third of villus enterocytes (data not shown).

DISCUSSION

In this study, copper deficiency is evidenced by depleted duodenal enterocyte copper concentration and consequently enterocyte copper-dependent oxidase activity. It also eliminated plasma ferroxidase I activity, produced anemia, and doubled hepatic iron stores. These findings support previous studies on the effect and extent of copper deficiency on iron metabolism in the rat and mouse (7, 8, 13, 14, 24, 25, 30, 33, 45). Because liver iron levels are raised in copper-deficient rats, it suggests that total iron absorption is increased; however, previous direct measurements have produced conflicting results (5, 7, 9). In this investigation, we demonstrate that copper deficiency increased iron ab-
It should be pointed out that in these studies, absorption of Fe(II) was measured; however, it cannot be excluded that absorption of Fe(III) is reduced by copper deficiency. This is based on the dependence of a surface-localized reductase such as Dcytb to reduce Fe(III) to Fe(II) before uptake. Yu and Wessling-Resnick (48) have shown that copper deficiency reduced uptake of Fe(III), possibly suggesting impairment of reductase activity.

These results therefore differ from those reported in copper-deficient swine, in which iron absorption is impaired and, supporting this, liver iron levels are lower than normal (17, 23). It is unlikely that in the swine, the reduced liver iron is due to increased release from hepatocytes, because parenteral iron leads to hepatocyte iron overload despite severe anemia (23). The most likely reason for this difference is greater dependence on copper-dependent ferroxidase activity in facilitating the release of iron from cells of swine compared with rats. In fact, rat and human Cp have significantly less biological activity than porcine Cp in increasing plasma iron levels in copper-deficient swine (35, 46) and in ferroxidase I activity (46). On the basis of the low ferroxidase activity of rat Cp, it was suggested that this function of Cp may be secondary to another more important function within Cp that exports iron from the liver (46).

It is also possible that Hp functions by means other than its ferroxidase activity. This is based on two observations. First, compared with other forms of stimulation of iron absorption, such as iron deficiency anemia and what occurs normally, with copper deficiency the mucosal iron levels and mucosal ferritin are low and appropriate for the increased level of iron absorption (see below). This suggests that there is no impairment to the release of iron from the enterocyte. Second, with copper deficiency, despite impaired Cp activity as evidenced by reduced plasma ferroxidase I activity and hepatocyte iron loading, mucosal iron levels are reduced, suggesting normal Hp activity. Thus, if the ferroxidase I activity of Hp is the main function of this protein, then enterocyte iron accumulation and impaired iron absorption would be seen. Because this was not observed, we suggest that in addition to copper-dependent ferroxidase activity, Hp may have another function that is lost by the mutation in sla mice. Recently, Syed and co-workers (37) modeled the NH2-terminus ectodomain of human Hp and suggested that the mutation to Hp found in sla mice would result in substantial nonfolding of the secondary structure of the protein that in turn would lead to impaired ferroxidase activity. However, it is also possible that another function residing within this region would also be compromised because of the mutation.

In copper-deficient rats, in contrast to increased iron levels within hepatocytes, macrophages have normal levels of iron as evidenced by normal Perl’s staining of Kupffer cells and normal spleen nonheme iron levels, a finding supported by others (7, 8, 24, 30). However, efflux of iron from the spleen clearly requires Cp activity, because in Cp knockout mice, spleen levels increased 250% compared with wild-type animals (18, 47). Thus it is possible that in the rodent, the efflux of iron from the macrophage also does not rely on ferroxidase I activity of Cp but on some other function performed by this protein.

The question as to whether there is impaired iron absorption during copper deficiency also needs to be considered from another perspective, namely the nature of the systemic regulators. The significantly increased iron absorption produced by copper deficiency is the sum of two major but opposing stimuli, namely increased erythropoiesis and increased liver iron absorption.

Fig. 3. Correlations between mucosal nonheme iron vs. mucosal ferritin (A) and total iron absorption (B). C: correlation between mucosal ferritin and total iron absorption. Data from normal (○), iron-deficient (●), and copper-deficient (□) animals were pooled to perform these analyses (n = 13). Correlation coefficients to these regressions are 0.93, −0.87, and −0.84, respectively. Mucosal nonheme iron concentration is expressed as % dose in body 5 days after gavage, and ferritin is expressed as arbitrary units/100 µg protein.

Fig. 4. Western blot analysis of protein from duodenal mucosa of copper-deficient animals (–Cu) and control (Con) animals. One hundred micrograms of protein were electrophoresed on an 8% polyacrylamide gel. After transfer, the gel was immunoreacted with polyclonal antibodies against human DMT1-IRE (A), rat ferroportin1 (B), and rat ferritin (C).
stores. The anemia of copper deficiency will stimulate the erythroid regulator and, in turn, this stimulates iron absorption. However, opposing this, increased iron stores, acting through the “stores” regulator possibly involving hepcidin, will inhibit iron absorption (15). Because the erythroid regulator is recognized to be the more potent of the two, a net but modest stimulation of iron absorption with copper deficiency was expected and found in this present study (15). To determine the effect of both of these stimulators operating cooperatively, we studied iron absorption in iron-deficient animals made anemic to a similar level as copper-deficient rats. These animals have increased erythropoiesis and markedly reduced hepatocyte iron stores. In addition, these animals also have markedly reduced enterocyte iron stores, which is another stimulator of iron absorption (4, 44). Under these circumstances, iron absorption was significantly raised above that of copper-deficient rats. In view of this comparison, it is likely that iron absorption in copper-deficient animals is operating appropriately for the level of stimulation from systemic regulators.

In summary, this study suggests in copper-deficient rodents that despite reduced copper-dependent ferrooxidase activity of duodenal enterocytes, iron absorption is unimpaired, whereas plasma ferrooxidase I activity is impaired, and there is hepatocyte iron accumulation. Macrophages also appear to rely on Cp for efficiency in iron absorption. In view of this, it will be of interest to determine whether the mutation to Hp in slax mice leads to the loss of its proposed copper-dependent ferrooxidase activity or of a yet-to-be-determined function that resides within the region deleted by the mutation.

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DISCLOSURES

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