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The effect of copper deficiency on the formation of hemosiderin in sprague-dawley rats

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Abstract We demonstrated previously that loading iron into ferritin via its own ferroxidase activity resulted in damage to the ferritin while ferritin loaded by ceruloplasmin, a copper-containing ferroxidase, was not damaged and had similar characteristics to native ferritin (Welch et al. (2001) Free Radic Biol Med 31:999–1006). Interestingly, it has been suggested that the formation of hemosiderin, a proposed degradation product of ferritin, is increased in animals deficient in copper. In this study, groups of rats were fed normal diets, copper deficient diets, iron supplemented diets, or copper deficient-iron supplemented diets for 60 days. Rats fed copper-deficient diets had no detectable active serum ceruloplasmin, which indicates that they were functionally copper deficient. There was a significant increase in the amount of iron in isolated hemosiderin fractions from the livers of copper-deficient rats, even more than that found in rats fed only an iron-supplemented diet. Histological analysis showed that copper-deficient rats had iron deposits (which are indicative of hemosiderin) in their hepatocytes and Kupffer cells, whereas rats fed diets sufficient in copper only had iron deposits in their Kupffer cells. Histologic evidence of iron deposition was more pronounced in rats fed diets that were deficient in copper. Additionally, sucrose density-gradient sedimentation profiles of ferritin loaded with iron in vitro via its own ferroxidase activity was found to have similarities to that of the sedimentation profile of the hemosiderin fraction from rat livers. The implications of these data for the possible mechanism of hemosiderin formation are discussed.

Keywords Ferritin · Ceruloplasmin · Hemosiderin · Copper · Iron

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Introduction

Iron can mediate the deleterious oxidation of many biomolecules if not tightly controlled. One mechanism of controlling the reactive nature of iron is to store excess iron in ferritin. Iron is

stored inside ferritin as a ferric oxyhydroxide polymer associated with some phosphate (deSilva et al. 1993; Granick and Hahn 1944). In order for iron to be incorporated into ferritin it must be presented as Fe(II) and subsequently oxidized to Fe(III). It remains uncertain as to how this occurs in vivo. Many researchers propose that the innate ferroxidase activity of the H chain of ferritin is responsible for iron incorporation (Bakker and Boyer 1986; Lawson et al. 1989; Theil 1990; Yang and Chasteen 1999). However, the stoichiometry of this reaction is 2:1, i.e., two moles of Fe(II) are oxidized per mole of oxygen reduced, indicating the production of H_2O_2 (Xu and Chasteen 1991). The production of H_2O_2 in the presence of Fe(II) could lead to the generation of the hydroxyl radical. The hydroxyl radical is a very potent oxidant capable of oxidizing ferritin or any other biomolecule in close proximity. In this regard, it has been demonstrated that the hydroxyl radical is indeed produced during iron loading into ferritin via its own ferroxidase activity (Grady et al. 1989). Ferritin loaded with iron via its own ferroxidase activity has different characteristics than native ferritin, including a number of oxidative modifications (Van Eden and Aust 2001; Welch et al. 2001). In this regard, the cysteine residues of ferritin have been shown to be integrally involved in the oxidation and subsequent aggregation of the ferritin (Welch et al. 2002).

Ceruloplasmin, a copper-containing ferroxidase, which catalyzes the oxidation of Fe(II) to Fe(III) with the concomitant reduction of oxygen to water (deSilva and Aust 1992; Ryden 1981) has also been shown to load iron into ferritin (Boyer and Schori 1983; deSilva et al. 1992; Guo et al. 1996; Juan et al. 1997). Interestingly, it has also been shown that there is a protein:protein interaction between ferritin and ceruloplasmin during iron loading (Juan and Aust 1998; Reilly et al. 1998). Importantly, ferritin loaded with iron via ceruloplasmin is not damaged and has similar characteristics to native ferritin (Welch et al. 2001). However, ceruloplasmin is generally considered a serum protein, while the loading of iron into ferritin occurs intracellularly. Therefore, questions regarding this method of loading iron into ferritin arise due to different compartmentalization of the proteins. However, a membrane-bound ferroxidase from cardiac tissue was shown to be capable of incorporating iron into ferritin (Reilly and Aust 1998). This intracellular ferroxidase was shown to also be a copper-containing enzyme (Reilly 1999). In addition, it was cross reactive with antibodies to ceruloplasmin, which indicates that it has a high similarity to ceruloplasmin.

In addition to ferritin, hemosiderin is considered by some researchers to be an iron storage protein. Ferritin has been extensively studied, and consequently the polypeptide structure and iron core of ferritin are well characterized. In addition, the mechanism by which ferritin synthesis is controlled is well known (Casey et al. 1988; Hentze et al. 1987). Hemosiderin, however, has not been studied as extensively. Relatively little is known about hemosiderin itself or its origin, even though this iron-protein complex is of fundamental importance in various iron-overload diseases (Selden et al. 1980). Hemosiderin is classically described as being composed of iron-rich, water-insoluble granules containing variable amounts of protein and is normally found in large membrane-bound aggregates or clumps (Iancu 1992). A number of reports have suggested that hemosiderin is a degradation product of ferritin (Miyazaki et al. 2002; O'Connell et al. 1986; O'Connell and Peters 1987). Studies have demonstrated a high similarity in the amino acid sequences of ferritin and the protein found in hemosiderin (Weir et al. 1984). Interestingly, it has been shown that hemosiderin has fewer aromatic and thiol residues than ferritin (Weir et al. 1984), suggesting that the ferritin was oxidized prior to conversion to hemosiderin.

A number of studies suggest that copper plays an integral role in the cellular iron homeostasis, due to the fact that defects in copper metabolism, as well as dietary-copper deficiency, have been shown to have profound effects on cellular and systemic iron homeostasis (Chen et al. 2006; Harris and Gitlin 1996; Lee et al. 1968; Roeser et al. 1970). Dietary-copper deficiency has been shown to induce an anemia that was not ameliorated by iron supplementation, however, the condition was completely reversed upon copper supplementation (Lee et al. 1968; Roeser et al. 1970). Additionally,

an involvement of ceruloplasmin in iron metabolism is evident from studies on individuals with aceruloplasminemia, a genetic disorder that results in a deficiency in active ceruloplasmin (Harris and Gitlin 1996; Okamoto et al. 1996; Takahashi et al. 1996; Yamaguchi et al. 1998). Aceruloplasminemia is characterized by hemosiderosis, diabetes mellitus, neurologic and ophthalmic degeneration, and altered pigmentation. Excessive iron deposition resembling hemosiderosis in liver, brain, and pancreas has been observed upon histological examination of tissues from patients diagnosed with aceruloplasminemia (Harris et al. 1995; Harris et al. 1998; Harris and Gitlin 1996; Yoshida et al. 1995). Similarly, patients with Wilson's disease, who have decreased serum ceruloplasmin ferroxidase activity, have also been shown to have hepatic hemosiderosis (Luca et al. 2000). Additionally, the Long-Evans Cinnamon rat, an animal model of Wilson's disease, has been shown to develop a fulminant hepatitis resulting from iron overload (Kato et al. 1996).

Researchers have suggested that animals deficient in copper have an increased amount of hemosiderin (Seo et al. 1996). Additionally, numerous studies have shown that animals fed iron supplemented diets have increased amounts of hemosiderin (Chua-Anusorn et al. 1999; Richter 1984; Whittaker et al. 1996). Therefore, it is possible that any condition which results in iron being loaded into ferritin via its own ferroxidase activity, either due to a lack of the proper ferroxidase or by overwhelming the ability of the ferroxidase to load iron into ferritin properly, may result in increased hemosiderin formation. This study was performed in an attempt to determine the mechanism by which copper deficiency causes an increase in hemosiderin formation. We provide data that copper-deficient rats have an unusual deposition of iron in their hepatocytes, indicating the formation of hemosiderin. We also provide data that copper deficiency results in an increased iron content of the hemosiderin fraction isolated from rat livers. Additionally, we demonstrated that these results are associated with a lack of an active copper-containing enzyme which has been shown to safely load iron into ferritin in vitro.

Materials and methods

Materials

Sodium chloride, potassium iodide, sucrose, and ferrous ammonium sulfate were purchased from Mallinckrodt. Tetramethyl ammonium hydroxide was purchased from Sigma. Phenylenediamine was purchased from Eastman-Kodak. All solutions were prepared in purified water (Barnstead NANOpure II system; specific resistance >17 mΩ/cm).

Proteins

Lyophilized human ceruloplasmin (EC 1.16.3.1), purchased from Vital Products, was resolubilized in 50 mM NaCl, pH 7.0, and was used within approximately 2 weeks to limit proteolysis (Ryan et al. 1992). Rat ferritin heavy (H) chain homomer was produced recombinantly using the same methods as described previously (Guo et al. 1996; Van Eden and Aust 2000).

Hemosiderin was isolated from rat liver as described by Ward et al. (1994) with modification. Briefly, approximately 50 g of liver tissue was minced and homogenized in 200 ml of 0.15 M NaCl, filtered through cheesecloth, and centrifuged at 8,000 g for 30 min. Next the pellet was washed with 100 ml of 0.15 M NaCl, centrifuged at 8,000 g for 30 min and resuspended in 4.1 M KI followed by centrifugation at 105,000 g for 2 h, twice (90 ml first and then 10 ml). Finally, the pellet was washed with H_2O (10 ml), centrifuged at 105,000 g for 1 h, and resuspended in 20 mM tetramethyl ammonium hydroxide (1 ml). Due to the fact that we had no way of verifying that all the protein obtained following this method for isolating hemosiderin was indeed hemosiderin, we use the term hemosiderin fraction, to refer to the insoluble, highly dense, iron-containing protein aggregate that was isolated.

Rat ferritin was isolated from the livers of Sprague-Dawley rats purchased from Pel-Freeze Biologicals. The entire purification process was performed at 4°C. Approximately 110 g of liver tissue was sliced and placed in 275 ml of 100 mM NaCl, pH 7.0, containing 20 mM

ε-amino-*n*-caproic acid and homogenized in a Potter-Elvehjem homogenizer. Sodium acetate (200 mM, pH 4.8) was added, while stirring, to the homogenate (25% v/v) and the pH was adjusted to 4.8 with glacial acetic acid. After 1 h of stirring, the mixture was centrifuged for 20 min at 1,500 g. Ammonium sulfate (35% w/V) was added to the supernatant and slowly stirred overnight. The mixture was centrifuged for 20 min at 2,500 g and the pellets containing ferritin were solubilized in ice-cold PBS, pH 7.0. The solubilized mixture was centrifuged at 30,000 g for 30 min, after which the supernatant was incubated at 65°C for 15 min. The heat-treated supernatant was centrifuged in a table-top centrifuge at 3,500 g for 15 min to remove all precipitated proteins. The supernatant was concentrated to 2 ml in an Amicon concentrator with an YM-100 filter membrane. The concentrated supernatant was chromatographed over a Sepharose CL-6B (Amersham Pharmacia Biotech) size-exclusion column pre-equilibrated with 50 mM NaCl, pH 7.0, and collected in 5 ml fractions. Ferritin rich fractions were determined by SDS-PAGE. The fractions containing ferritin were concentrated and chromatographed over a Bio-Gel A-15 m (BioRad) size-exclusion column. Fractions containing ferritin were once again identified by SDS-PAGE analysis, pooled and concentrated to 1 ml.

Animals and diets

Twenty male weanling Sprague-Dawley rats were purchased from Harlan Sprague-Dawley. Throughout the study, rats had ad lib access to food and water. After a 7-day accumulation period, rats were randomly separated into four treatment groups. The groups received one of four dietary treatments for 60 days (five rats per group). Diets (Harlan Teklab Laboratories) consisted of a standard purified diet, a copper-deficient diet, an iron-supplemented diet, and a copper deficient/iron supplemented diet. The copper-deficient diets contained 0.43 mg Cu/kg, the iron-supplemented diets contained 700 mg Fe/kg, and the standard purified diet contained 10.5 mg Cu/kg and 210 mg Fe/kg. This study was approved by the Utah State University Laboratory Animal Care and Use Committee.

Rats were killed using CO_2 after which they were exsanguinated by cardiac puncture. Liver, spleen, heart, duodenum, and partial jejunum were collected and fixed in 10% neutral-buffered formalin. Tissue specimens were trimmed, processed, embedded in paraffin, and sectioned at 5 μm. One set of each tissue was stained with hematoxylin and eosin (H&E) for conventional histopathology evaluation and one set with Perl's stain for iron. Tissues were scored from 1 to 4, with 4 indicating the greatest number of stained foci of iron deposition. Ten randomly selected view fields were scored for each animal at 200× magnification. The number of stained foci of iron deposits in Kupffer cells and hepatocytes were separately scored in the same view fields. Kupffer cell iron accumulation was scored as follows: (1) no visible iron staining, (2) iron staining in ≤ 5 Kupffer cells per view field, (3) iron staining in more than 5 but ≤ 10 Kupffer cells per view field, and (4) iron staining in more than 10 Kupffer cells per view field. Hepatocyte iron accumulation was scored as follows: (1) no visible iron staining, (2) iron staining in ≤ 10% of hepatocytes per view field, (3) iron staining in more than 10% but ≤ 50% of hepatocytes per view field, and (4) iron staining in more than 50% of hepatocytes per view field.

p-Phenylenediamine oxidase assay

Sera were analyzed for *p*-phenylenediamine oxidase activity as an indicator of ceruloplasmin activity (Ravin 1961). Briefly, 20 μl of serum was incubated with 10 mM *p*-phenylenediamine in 100 mM sodium acetate, pH 5.0, at 37°C. Oxidation of *p*-phenylenediamine was determined spectrophotometrically using an EL311 microplate reader (BioTek Instruments) using dual-wavelength with detection at 570 nm and background subtraction at 630 nm. One unit of activity was defined as the amount of oxidase required to cause an absorbance change of 1.0 per hour at 570 nm.

Iron loading assays

Loading iron into ferritin was performed as described previously (deSilva et al. 1992), with

modifications. Briefly, reaction mixtures containing 2 mg ferritin were incubated with ferrous ammonium sulfate in 50 mM NaCl, pH 7.0, at 37°C. Four additions of iron were added in increments of 250 molar equivalents (Fe(II): ferritin) at 20-min intervals. The iron content of proteins was determined using the method described by Brumby and Massey (1967).

Sucrose density-gradient centrifugation

Samples of ferritins or hemosiderin were placed on top of a 10 ml continuous sucrose gradient (1–25% w/v) and centrifuged in a Beckman SW41Ti swinging bucket rotor at 30,000 rpm (~110,000 g) for 2.5 h. Aliquots (1 ml) were removed from the top of the gradient by pipette and analyzed for protein content by the Bradford Assay.

Mineral analyses

Liver copper and iron analyses were performed on nitric acid digested samples. Briefly, liver samples were dried to a constant weight, and then digested with 1 ml of trace mineral grade nitric acid (Fisher Scientific) in sealed 10-ml Oak Ridge Teflon digestion tubes (Nalge Nunc International) for 1 h at 90°C. A 0.5 ml aliquot of the digest was added to 9.5 ml of 18.3 mΩ water in a 15-ml polypropylene trace metal free tube (ELKAY). This provided a 5% nitric acid matrix for the analysis, which was matrix matched for all standard curve and quality control samples. Mineral content analysis was performed using an ELAN 6000 inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer). For both elements, five point standard curves from 0.01 to 0.50 mg/l were used to quantify the minerals. Sequential 1:10 dilutions were made, using 5% nitric acid, for minerals exceeding the standard curve. Standard curves and quality control samples were analyzed every five samples. NIST standards also were analyzed to verify accuracy of the analytical results.

Statistics

The results are presented as means ± SD, the significance of differences between treatments was determined using ANOVA with a posthoc test (Fisher LSD Method) of significance between individual groups. Differences were considered significant when $P < 0.05$.

Results

The effect of copper deficiency on the *p*-phenylenediamine oxidase activity of sera

There was no detectable *p*-phenylenediamine oxidase activity in the sera of rats consuming diets deficient in copper (Table 1), even when the volume of serum assayed was increased four-fold (data not shown). Rats consuming diets with a sufficient amount of copper had approximately 14 U/ml of activity. By comparing the amount of *p*-phenylenediamine oxidase activity of rat serum

Table 1 The effect of copper deficiency on animal weights and *p*-phenylenediamine oxidase activity of rat sera

Group	Animal weights(g)	*p*PD oxidase activity (Units/ml serum)	Serum ceruloplasmin (mg/ml)[a]
Control	346 ± 22	14.1 ± 1.6	1.1 ± 0.1
Copper deficient	333 ± 30	ND	ND
Iron supplemented	347 ± 26	12.5 ± 4.7	0.9 ± 0.4
Copper deficient/ iron supplemented	331 ± 20	ND	ND

Oxidase activity was determined using the *p*-phenylenediamine assay as described under Materials and Methods. Values are the means ± SD for five rats per group.

ND: No *p*-phenylenediamine oxidase activity was detected.

[a] Serum ceruloplasmin concentrations of rat sera were estimated by comparing the *p*-phenylenedamine oxidase activity of the rat sera to that of pure human ceruloplasmin (Ryan et al. 1992)

Table 2 The effect of copper deficiency and iron supplementation on hepatic iron and copper concentrations

Group	Hepatic copper concentration (ppm)*	Hepatic iron concentration (ppm)*
Control	12.8 ± 2.3[b, d]	248 ± 62[b, c, d]
Copper deficient	7.3 ± 2.0[a, c]	572 ± 104[a]
Iron supplemented	16.3 ± 7.3[b, d]	534 ± 124[a]
Copper deficient/ iron supplemented	6.9 ± 1.7[a, c]	674 ± 233[a]

* The concentration of hepatic iron and copper was determined using inductively coupled plasma-mass spectrometry (ICP-MS) from dry liver tissue. The data represent the means ± SD for five rats per group.

[a] Value significantly different from that for rats fed control diet: $P < 0.05$.

[b] Value significantly different from that for rats fed copper deficient diet: $P < 0.05$.

[c] Value significantly different from that for rats fed iron supplemented diet: $P < 0.05$.

[d] Value significantly different from that for rats fed copper deficient/iron supplemented diet: $P < 0.05$

to that of pure human ceruloplasmin, we estimated the serum concentration of active rat ceruloplasmin to be approximately 1 mg/ml.

The rats consuming copper-deficient diets had no detectable active serum ceruloplasmin, which is a good indication that they were copper deficient. Additionally the concentration of hepatic copper was significantly lower in the rats consuming copper-deficient diets, while their hepatic iron concentrations were higher (Table 2). The overall health of all four groups of rats was similar and the difference in the group averaged weights of the animals was not statistically significant with respect to diet (Table 1). However, the difference in weights was increasing with time (data not shown), which suggests that if the study had been extended, the difference in weights likely would have become statistically significant.

Total iron analysis of the hemosiderin fraction

Hemosiderin was isolated from the livers of the rats in each group. Protein was detected in the hemosiderin fraction from each group. The hemosiderin fraction isolated from the control group had a minimal amount of iron (Fig. 1). Whereas, the hemosiderin fraction isolated from the remaining three groups contained large amounts of iron. Interestingly, the hemosiderin fraction isolated from both groups fed copper-deficient diets had significantly more iron than the hemosiderin fraction from the control and iron supplemented groups.

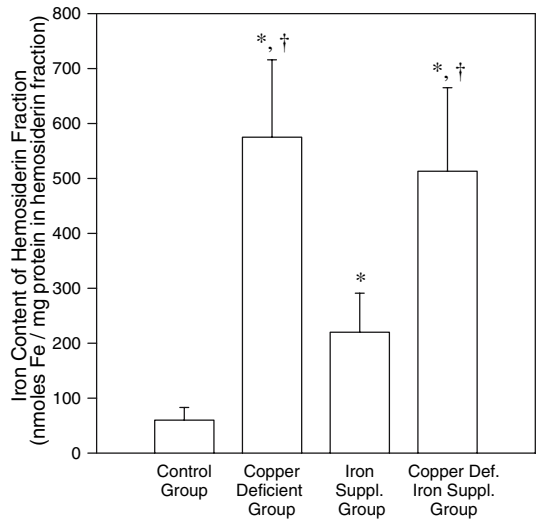

Fig. 1 The effect of copper deficiency and iron supplementation on the iron content of the hemosiderin fraction. Iron content of hemosiderin fraction (*n*moles) per mg of protein. Results represent means ± SD ($n = 5$ mice per group); $^{*}P < 0.05$ compared to control diet group; $^{\dagger}P < 0.05$ compared to iron-supplemented diet group

Histological analysis of tissues

Histological evaluation of the tissues stained with hematoxylin and eosin (H&E) showed no discernable tissue damage in any of the rats. Additionally, no iron deposits were observed in heart, duodenum, and jejunum tissues stained for iron in any of the rats. However, in both liver and spleen there was an increased amount of iron deposits in the rats fed either copper-deficient and/or

iron-supplemented diets. Although differences were noticeable in the spleens, we were unable to quantitate the visual differences due to extremely high variability among view fields. The iron deposits were localized to the sinuses of the spleens in all groups (data not shown).

The differences in iron deposition in the livers of the rats are shown in Fig. 2. The rats fed control diets had little or no indication of iron deposits. Any deposits that were observed were seen strictly in Kupffer cells. The rats fed the iron-supplemented diet also had iron deposits in Kupffer cells, with significantly more iron deposits than that seen in the rats fed the control diet (Table 3). The rats fed diets deficient in copper also had iron deposits in Kupffer cells. Additionally, a very interesting finding was that the rats fed copper-deficient diets also had iron deposits in their hepatocytes. However, only in the rats fed a diet deficient in copper and supplemented with iron was iron deposition in both Kupffer cells and hepatocytes significantly different from the rats fed the control diet.

Sucrose density gradient sedimentation profiles of the hemosiderin fraction and ferritins

Due to the fact that hemosiderin is reported to be a degradation product of ferritin the protein in the hemosiderin fraction isolated from rat livers was compared to ferritins damaged by the loading of iron via their own ferroxidase activity. The sucrose-density gradient sedimentation profile of the hemosiderin fraction was compared to those of recombinant rat ferritin loaded with iron via its own ferroxidase activity and native rat liver ferritin (Fig. 3). Almost all of the native rat liver ferritin was detected in the first seven fractions of the sucrose gradient, with essentially no protein detected in the last five fractions. Conversely, approximately 60% of the protein in the hemosiderin fraction loaded onto the sucrose gradient sedimented completely through the gradient. Similarly, approximately 10% of recombinant rat ferritin loaded with iron via its own ferroxidase

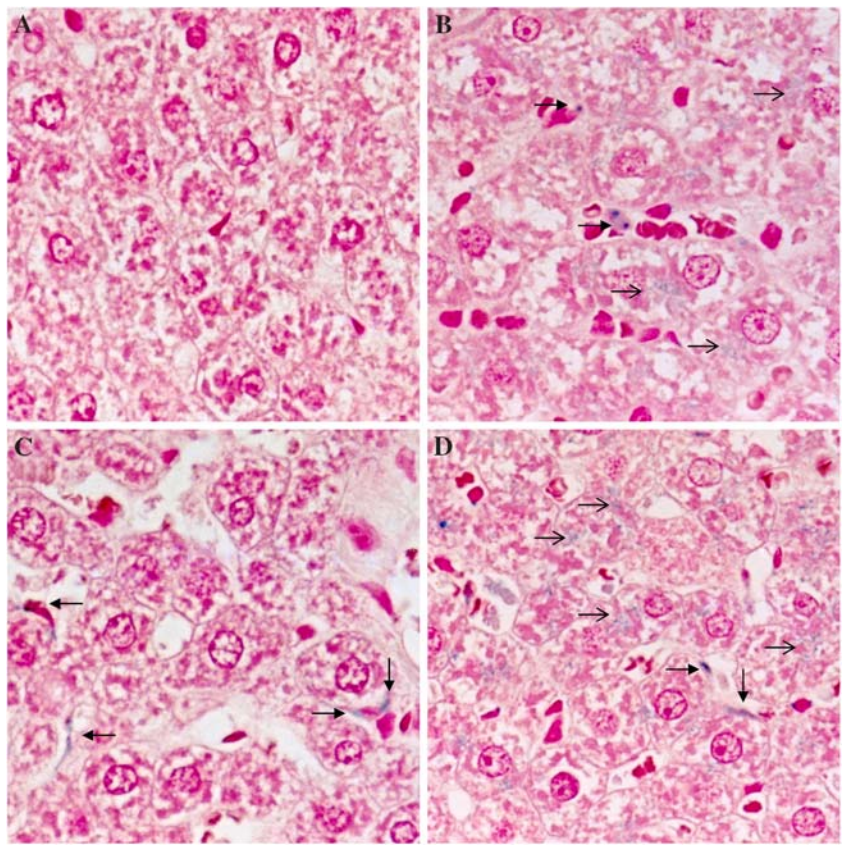

Fig. 2 The effect of copper deficiency and iron supplementation on hepatic iron deposition. Pictures are of liver sections stained for iron. (**A**) Rats fed a control diet, (**B**) rats fed a copper-deficient diet, (**C**) rats fed an iron-supplemented diet, (**D**) rats fed a copper-deficient/iron-supplemented diet. Bold arrows indicate iron deposits in Kupffer cells and the regular font arrows indicate iron deposits in hepatocytes

Table 3 The effect of copper deficiency and iron supplementation on hepatic iron deposition

Group	Iron deposition in Kupffer cells*	Iron deposition in hepatocytes*
Control	1.0 ± 0.0[c, d]	1.0 ± 0.0[d]
Copper deficient	1.3 ± 0.2[d]	1.2 ± 0.3[d]
Iron supplemented	1.8 ± 0.3[a, d]	1.0 ± 0.1[d]
Copper deficient/iron supplemented	2.4 ± 0.7[a, b, c]	2.8 ± 1.2[a, b, c]

* The data represent the visualization of stained iron deposits which was determined by average scoring of 10 view fields per rat. Iron deposition was scored from 1 to 4, with 1 indicating no stained foci and 4 indicating the greatest number of stained foci per view field. The data represent the means ± SD for five rats per group.

[a] Value significantly different from that for rats fed control diet: $P < 0.05$.

[b] Value significantly different from that for rats fed copper deficient diet: $P < 0.05$.

[c] Value significantly different from that for rats fed iron supplemented diet: $P < 0.05$.

[d] Value significantly different from that for rats fed copper deficient/iron supplemented diet: $P < 0.05$

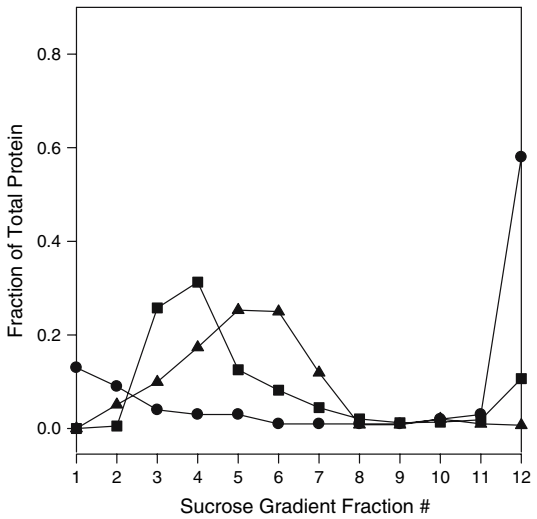

Fig. 3 Comparison of the sucrose gradient sedimentation profiles of hemosiderin, recombinant rat ferritin loaded with iron via its own ferroxidase activity, and native rat liver ferritin. (●) Hemosiderin fraction, (■) recombinant rat ferritin loaded with iron via its own ferroxidase activity, (▲) native rat liver ferritin. Aliquots (1 ml) were removed from the top (fraction #1) of each gradient by pipette and analyzed for protein. The data are representative of 2–3 sedimentation profiles for each group

activity sedimented completely through the sucrose-density gradient.

Discussion

A relationship between copper and iron metabolism has been known for some time (Chen et al. 2006; Harris and Gitlin 1996; Lee et al. 1968; Roeser et al. 1970). However, the mechanism(s) by which copper is involved in iron metabolism remains largely unknown. It is possible that a copper-containing enzyme, such as ceruloplasmin, may play a key role in iron metabolism due to its ferroxidase activity and its ability to load iron into ferritin (deSilva and Aust 1992; Guo et al. 1996; Juan et al. 1997; Juan and Aust 1998; Reilly et al. 1998; Reilly 1999; Reilly and Aust 1998). Although, it remains unknown how iron is loaded into ferritin in vivo, considerable data have been presented indicating that when iron is loaded into ferritin in vitro via its own ferroxidase activity the ferritin is damaged (deSilva et al. 1992; Van Eden and Aust 2001; Welch et al. 2001, 2002). This is intriguing because hemosiderin is thought to be a degradation product of ferritin (Miyazaki et al. 2002; O'Connell et al. 1986; O'Connell and Peters 1987). Additionally, one of the symptoms of aceruloplasminemia, a disorder in which patients lack active ceruloplasmin, is an increase in hemosiderin formation (Harris and Gitlin 1996). Therefore, our hypothesis is that ceruloplasmin or a ceruloplasmin-like (copper-containing) enzyme is required to safely load iron into ferritin. Consequently any abnormality such as aceruloplasminemia, copper deficiency, or iron-overload diseases, which could result in either an inability to load iron into ferritin properly due to a lack of the proper enzyme, or by overloading the capability of the enzyme to load iron into ferritin, may result in an increased amount of oxidized ferritin, which could then be processed into hemosiderin.

In this study there were no significant effects from copper deficiency on the health or weight gain of the rats, which indicates that the differences observed in the study are not due to underlying discrepancies in the health of the rats, as they were all healthy. However, the lack of a difference in weight gain due to copper deficiency is contradictory to other reports (Prohaska and Brokate 2001; Williams et al. 1983). This discrepancy may be explained by the fact that the rats received some copper via their drinking water. The rats were supplied with regular tap water, which had approximately 0.2 ppm copper and 0.4 ppm iron. In total, the rats fed copper-deficient diets received 0.7 mg of copper in the 60 day treatment (half of which came from their drinking water) versus the 13.2 mg of copper that the rats fed copper-sufficient diets received. Although, 0.7 mg of copper is significantly less copper than the control group received, it may have been enough to allow the rats to grow well for 60 days. However, the rats fed a copper-deficient diet were indeed copper-deficient, as indicated by both their decreased concentration of hepatic copper (Table 2) and their lack of *p*-phenylenediamine oxidase activity in their sera, which is a good indication of the copper status of the animals (Kehoe et al. 2000), because the primary enzyme responsible for oxidizing *p*-phenylenediamine in serum is the copper-containing enzyme ceruloplasmin (Ravin 1961).

Although the procedure for isolating hemosiderin has been published (Ward et al. 1994; Weir et al. 1984), and it is thought that hemosiderin is a degradation product of ferritin (Miyazaki et al. 2002; O'Connell et al. 1986; O'Connell and Peters 1987), it remains unknown if all of the protein obtained upon collecting the hemosiderin fraction is indeed oxidized and degraded ferritin. We did not find a correlation between the diets the animals received and the amount of protein in the hemosiderin fraction. However, as reported in Fig. 1, there was a correlation between the diets and the iron content of the hemosiderin fraction, with copper deficiency having the most pronounced effect. These data indicate that copper deficiency alters the iron metabolism of the animal such that more iron is found in the hemosiderin fraction. This could potentially be a result of an inability to properly load iron into ferritin, thus damaging the ferritin and consequently causing more ferritin to be converted to hemosiderin.

Histological evaluation of the livers (Fig. 2) seems to corroborate the hypothesis that copper deficiency increases hemosiderin formation. In copper-sufficient rats very few iron deposits were observed, and they were only observed in Kupffer cells, where it is not uncommon to see iron deposition even under normal conditions. Interestingly, even in the rats receiving iron-supplemented diets, the iron deposits were only observed in the Kupffer cells. However, in the rats receiving copper-deficient diets there were small deposits of iron observed in their hepatocytes. The rats receiving a copper-deficient and iron-supplemented diet had even more iron deposits in their hepatocytes. Similarly, in field cases of goats that were severely copper deficient (< 1 ppm copper in their livers), iron deposits were observed in Kupffer cells and hepatocytes (unpublished data). These data indicate that exposing the animals to excess iron in a compromised state, such as copper deficiency, could be even more detrimental to the animal due to its inability to properly process the iron.

Hemosiderin by definition is an insoluble aggregate of protein and iron (Iancu 1992), and therefore it is not surprising that the majority of the hemosiderin fraction sedimented completely through the sucrose gradient and pelleted on the bottom of the tube (Fig. 3). In contrast, the native rat liver ferritin only sedimented into the top two thirds of the sucrose gradient. Interestingly, the recombinant rat ferritin loaded in vitro via its own ferroxidase activity had sedimentation characteristics of both native ferritin, in that the majority of the protein was detected in the top half of the gradient, and hemosiderin, in that ~10% of the protein sedimented through the gradient and pelleted on the bottom of the centrifuge tube Our hypothesis is that the 10% of the protein that sedimented on the bottom of the centrifuge tube, is the ferritin that has been sufficiently oxidized/modified such that it is subsequently processed by the cell to form hemosiderin. Interestingly, this process may occur even more readily in humans because human ferritin appears to be more susceptible than rat ferritin to the oxidative

modifications that can occur when iron is incorporated via its own ferroxidase activity (unpublished observations). One of the few differences in the H subunit of rat and human ferritins is that rat ferritin has an arginine residue at position 90, whereas human ferritin has a cysteine residue. In this regard, we showed previously that substituting the cysteine residue at position 90, for the arginine residue significantly prevented the oxidation and aggregation of human ferritin (Welch et al. 2002). Consequently, copper deficiency in humans may play an even more profound role in the formation of hemosiderin.

In conclusion, we demonstrated in this study that copper deficiency does indeed alter iron metabolism, such that there is an abnormal deposition of iron in the hepatocytes of copper-deficient rats and that the iron content of the hemosiderin fraction was significantly increased in copper-deficient rats. Additionally, we demonstrated that copper-deficient rats were deficient in an enzyme, ceruloplasmin, which is known to play vital roles in iron metabolism, perhaps even the loading of iron into ferritin in vivo. Therefore, we suggest that the mechanism by which copper deficiency results in increased hemosiderin formation could be due to an inability to properly load iron into ferritin due to a lack of the copper-containing enzyme responsible for safely loading iron into ferritin.

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