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RESEARCH PAPER

Suppressed hepcidin expression correlates with hypotransferrinemia in copper-deficient rat pups but not dams

Margaret Broderius · Elise Mostad · Joseph R. Prohaska

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Abstract Copper deficiency leads to anemia but the mechanism is unknown. Copper deficiency also leads to hypoferremia, which may limit erythropoiesis. The hypoferremia may be due to limited function of multi-copper oxidases (MCO) hephaestin in enterocytes or GPI-ceruloplasmin in macrophages of liver and spleen whose function as a ferroxidase is thought essential for iron transfer out of cells. Iron release may also be limited by ferroportin (Fpn), the iron efflux transporter. Fpn may be lower following copper deficiency because of impaired ferroxidase activity of MCO. Fpn is also dependent on the liver hormone hepcidin as Fpn is degraded when hepcidin binds to Fpn. Anemia and hypoferremia both down regulate hepcidin by separate mechanisms. Current studies confirmed and extended earlier studies with copper-deficient (CuD) rats that suggested low hepicidin resulted in augmented Fpn. However, current studies in CuD dams failed to confirm a correlation that hepcidin expression was associated with low transferrin receptor 2 (TfR2) levels and also challenged the dogma that holotransferrin can explain the correlation with hepcidin. CuD dams exhibited hypoferremia, low liver TfR2, anemia in some rats, yet no depression in *Hamp* expression, the hepcidin gene. Normal levels of GDF-15, the putative erythroid cytokine that suppresses hepcidin, were detected in plasma of CuD and iron-deficient (FeD) dams. Importantly, FeD dams did display greatly lower *Hamp* expression. Normal hepcidin in these CuD dams is puzzling since these rats may need extra iron to meet needs of lactation and the impaired iron transfer noted previously.

Keywords Copper deficiency · Rats · Transferrin iron · Hepcidin · Anemia

M. Broderius · E. Mostad · J. R. Prohaska (✉)
Department of Biomedical Sciences, University of Minnesota Medical School Duluth, 1035 University Drive, Duluth, MN 55812, USA
e-mail: jprohask@d.umn.edu

Introduction

Limitation in the availability of the essential trace metal copper leads to a series of pathological consequences because copper is a required cofactor for about a dozen enzymes (Prohaska 2012). One such consequence is anemia reflected in a severe drop in erythrocyte cell number and lower hemoglobin content per cell. Copper supplementation can reverse this anemia as first documented in 1862 (Fox 2003). The precise mechanism for the anemia of copper deficiency is not known but is more complicated than the original ferroxidase hypothesis (Osaki et al. 1966). Copper-deficient (CuD) rats and other species exhibit hypoferremia with anemia (Cartwright et al. 1956). Since ceruloplasmin (ferroxidase) null mice (*Cp* −/−) also exhibit hypoferremia, it seems logical to conclude that lack of copper can limit Cp function or other multicopper oxidases (MCO) function that impact iron efflux (Prohaska 2011).

However, iron efflux also depends on ferroportin (Fpn), the only known and essential iron efflux translocator (Donovan et al. 2005). It is believed that Fpn works in concert with tissue-specific MCO enzymes to convert ferrous to ferric iron to load transferrin (Tf), the plasma iron transport protein (Collins et al. 2010). Plasma membrane abundance of Fpn has been shown to depend on the ferroxidase activity of Cp (De Domenico et al. 2007). Thus,

hypoferremia of CuD rats might be caused by lower MCO function or lower Fpn abundance.

Some believe that the abundance of Fpn is determined largely by hepcidin (Hepc), a liver hormone that binds Fpn and results in internalization and degradation (Nemeth et al. 2004). When Hepc is produced, iron absorption from the intestine and iron mobilization from macrophage stores are decreased by the destruction of Fpn. Hepc expression is regulated by a complex set of factors that include holo-transferrin, IL-6, bone morphogenic protein 6 (BMP6), and putative factors that are released from bone marrow in response to anemia (Knutson 2010).

Recent studies in male CuD rat pups and postweanling male rats reported a profound reduction in *Hamp* expression (gene coding for hepcidin) (Jenkitkasemwong et al. 2010). These CuD rats had lower hemoglobin and plasma iron levels than copper-adequate (CuA) controls. The lower holotransferrin (plasma iron) and lower expression of transferrin receptor 2 (TfR2), a sensor of plasma iron, correlated well with lower *Hamp* expression. *BMP6* levels were not impacted by CuD in that study (Jenkitkasemwong et al. 2010). Augmented liver iron can also stimulate *Hamp* expression (Knutson 2010). In the CuD rat studies mentioned, liver iron levels were higher in CuD than CuA rats but this did not correlate with *Hamp* expression.

Recently, a modification to the commonly used AIN-76A and AIN-93G diets has allowed development of CuD rats without augmentation in liver iron (Bastian et al. 2011). In fact, previous work with AIN-76A diet suggests it contained lower dietary iron, approximately 50 mg Fe/kg, than needed to support hemoglobin development in pups (Pyatskowit and Prohaska 2008b). Further, the new modified AIN-93G diet, approximately 80 mg Fe/kg, resulted in augmented liver iron in both CuA male pups and their dams compared to AIN-76A diet (Bastian et al. 2011).

The purpose of the current studies was to evaluate *Hamp* expression in CuD female pups reared on the AIN-76A diet, in CuD male pups, CuD dams, and iron-deficient (FeD) dams reared on the new modified AIN-93G diets to elucidate the signaling pathway in CuD rats that may impact *Hamp* expression and iron homeostasis.

Materials and methods

Animal care and diets

In experiment one (Expt. 1), 10 Holtzman sperm-positive rats were purchased commercially (Harlan Sprague–Dawley, Indianapolis, IN) and received either Cu-adequate (CuA) or Cu-deficient (CuD) dietary treatment consisting of a Cu-deficient modified AIN-76A diet (Teklad Laboratories, Madison, WI) containing 0.4 mg Cu/kg and 50 mg Fe/kg. Normal AIN-76A diet contains approximately 6 mg Cu/kg. All dams and offspring were fed the CuD diet. CuA groups drank water supplemented with cupric sulfate, 20 mg Cu/L, and CuD groups drank Cu-free deionized water. Treatment for dams began on embryonic day 7, and all rats were killed after 5 weeks of treatment.

In Expt. 2, 15 Sprague–Dawley sperm-positive rats were purchased commercially (Charles River, Willmington, MA, USA) and received either CuA, CuD, or FeD diets modified from the AIN-93G formulation by increasing dietary iron, copper, calcium, and phosphorous to those recommended for reproduction listed in Table 2–2 of the 1995 National Research Council guidelines for rats, described elsewhere (Bastian et al. 2010). All dams were offered deionized water to drink. CuD diet contained 0.5 mg Cu/kg and 78 mg Fe/kg; FeD diet contained 7.2 mg Cu/kg and 5.3 mg Fe/kg; CuA diet contained 8.7 mg Cu/kg and 80 mg Fe/kg. Treatment for dams began on embryonic day 2. All dams were killed after 6 weeks of treatment. Nutritional protocols are similar to recent designs (Bastian et al. 2011).

For Expt. 1, one female pup from each litter was sampled on postnatal day 26 (P26), and for Expt. 2, one male pup P25 from CuA and CuD dams was sampled. FeD pups in Expt. 2 did not survive to P25. All rats were maintained at 24 °C with 55 % relative humidity on a 12-h light cycle (0700–1900 h). All protocols were formally approved by the University of Minnesota Institutional Animal Care and Use Committee.

Tissue collection

To prevent potential changes induced by anesthetics, pups were decapitated without anesthesia. Upon decapitation, trunk blood was allowed to clot and serum was harvested and stored at −75 °C until analysis. An aliquot of blood was used to measure hemoglobin. Liver and spleen were rapidly dissected and a portion used for metal analyses.

Dams were deeply anesthetized with either ketamine/xylazine (Expt. 1) or carbon dioxide (Expt. 2) prior to cardiac puncture. Plasma was harvested, and an aliquot of liver and spleen was used for metal analyses. Remaining tissue was rapidly frozen and stored at −75 °C until analysis.

Biochemical analyses

Diet samples and liver tissues were wet-digested with HNO_3 (Trace Metal grade; Fisher Scientific, Pittsburgh, PA, USA), and samples were analyzed for total copper, iron, and zinc content by flame atomic absorption spectroscopy (AAS) (Model AA240 FS, Varian, Walnut Creek, CA, USA) (Pyatskowit and Prohaska 2008a). Plasma or

serum was analyzed for iron content by AAS following treatment with hot trichloroacetic acid to remove hemoglobin iron as described previously (Pyatskowit and Prohaska 2008a). Hemoglobin was determined spectrophotometrically at 540 nm after conversion to metcyanhemoglobin (Prohaska 1991). Spleen non-heme iron (NHI) was determined colorimetrically after acid digestion using a modification of an established protocol (Torrance and Bothwell 1968).

PCR methods

Quantitative real-time PCR was used to evaluate mRNA content of selected hepatic genes following suggested guidelines (Bustin et al. 2009). Total RNA was isolated from liver tissue and quality established spectrophotometrically and by RNA gels (Prohaska and Brokate 1999). The relative mRNA content of hepatic hepcidin (*Hamp*), ferroportin (*Fpn*), and bone morphogenic protein 6 (*Bmp6*) were determined by qPCR using the reference gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as described previously (Broderius et al. 2010). Primer pairs for Hamp were 5′ GGC AGA AAG CAA GAC TGA TGA C 3′ (forward) and 5′ ACA GGA ATA AAT AAT GGG GCG 3′ (reverse); for *Fpn* 5′ GGT GGT GGC AGG CTC TGT 3′ (forward) and 5′ TTT GAA CCA CCA GGG ACG TC 3′ (reverse); for *Bmp6* 5′ ATG GCA GGA CTG GAT CAT TGC 3′ (forward) 5′ CCA TCA CAG TAG TTG GCA GCG 3′ (reverse); and for *Gapdh* 5′ TTC CTA CCC CCA ATG TAT CCG 3′ (forward) and 5′ ACC ACC CTG TTG CTG TAG CCA 3′ (reverse).

Western immunoblot protocols

Pieces of fast frozen liver and spleen were homogenized and used to prepare membranes, and proteins were solubilized as described previously (Mostad and Prohaska 2011). Membrane proteins were mixed with SDS Laemmli sample buffer without boiling for Fpn or boiled for all other proteins and electrophoretically fractionated by SDS–PAGE on 10 % gels. Spleen cytosolic samples were treated similarly, but first boiled for 5 min and fractionated on 15 % gels. Plasma proteins were fractionated on 10 % gels. It was only possible to analyze three plasma samples of each treatment group from Expt. 2 for GDF-15 because a thicker comb and larger plasma volume were required. Separated proteins were transferred to a Protran BA 83 nitrocellulose membrane (Whatman) and treated as described previously to detect the appropriate antigens (Broderius et al. 2010). Membranes were stained with Ponceau S to ensure equal loading of protein. Immunoreactivity was visualized using SuperSignal chemiluminescence substrate (Thermo Scientific). Chemiluminescence detection and densitometry were carried out using the FluorChem™ system (Alpha Innotech, San Leandro, CA, USA).

The following antibodies were those used previously or purchased commercially and used at appropriate dilutions: anti-mouse actin (MAB1501, Millipore), mouse anti-human transferrin receptor (TfR1) (13-6800, Zymed Laboratories), goat anti-human ceruloplasmin (C 0911, Sigma-Aldrich), goat anti-mouse GDF-15 (sc-46248, Santa Cruz Biotechnology), goat anti-human transferrin (T 6265, Sigma-Aldrich), rabbit anti-scavenger receptor B1 (SRB1) (NB400-104, Novus Biologicals), and rabbit anti-transferrin receptor 2 (TfR2) (sc-48747, Santa Cruz Biotechnology). Affinity-purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and rabbit anti-rat/mouse ferroportin (Fpn) were characterized previously (Jenkitkasemwong et al. 2010; West and Prohaska 2004).

Statistical analyses

Dietary treatment effects were evaluated by Student's *t* test after variance equality was tested by *F* test, $\alpha = 0.05$. Dams data in Expt. 2 were analyzed by one-way ANOVA and Tukey's test. Data were analyzed using Microsoft Excel™ or KaleidaGraph (Synergy Software, Reading, PA, USA). For immunoblot densitometry, a value of 1.0 was assigned to mean pixel density of CuA samples and then relative individual densities were recalculated. Some other data were normalized for graphic presentation.

Results

Copper and iron status of rats

Four experimental groups were evaluated after treatment with CuD or FeD diets. Female and male CuD pups had liver copper levels less than 10 % of their CuA controls (Fig. 1a). CuD dams fed the modified AIN-76A diet had markedly lower liver copper as well (Expt. 1 Dams1). CuD dams fed the modified AIN-93G diet with extra iron, calcium, and phosphorus also had lower liver copper levels than CuA dams (Expt. 2 Dams2), but liver copper in FeD dams was augmented by 50 % (Fig. 1a).

Liver iron level was not altered by either CuD diet in rat pups (Fig. 1b). Liver iron was higher in CuD Dams1 than CuA Dams1; however, liver iron was not augmented in CuD dams fed the new CuD diet. As expected, liver iron was greatly lower in FeD dams averaging 1.88 μg/g for five dams compared 179 μg/g for CuA dams.

Hemoglobin concentration was lower in all CuD groups compared to CuA controls except CuD Dams2 (Fig. 1c). Mild anemia was observed in FeD dams in this experiment

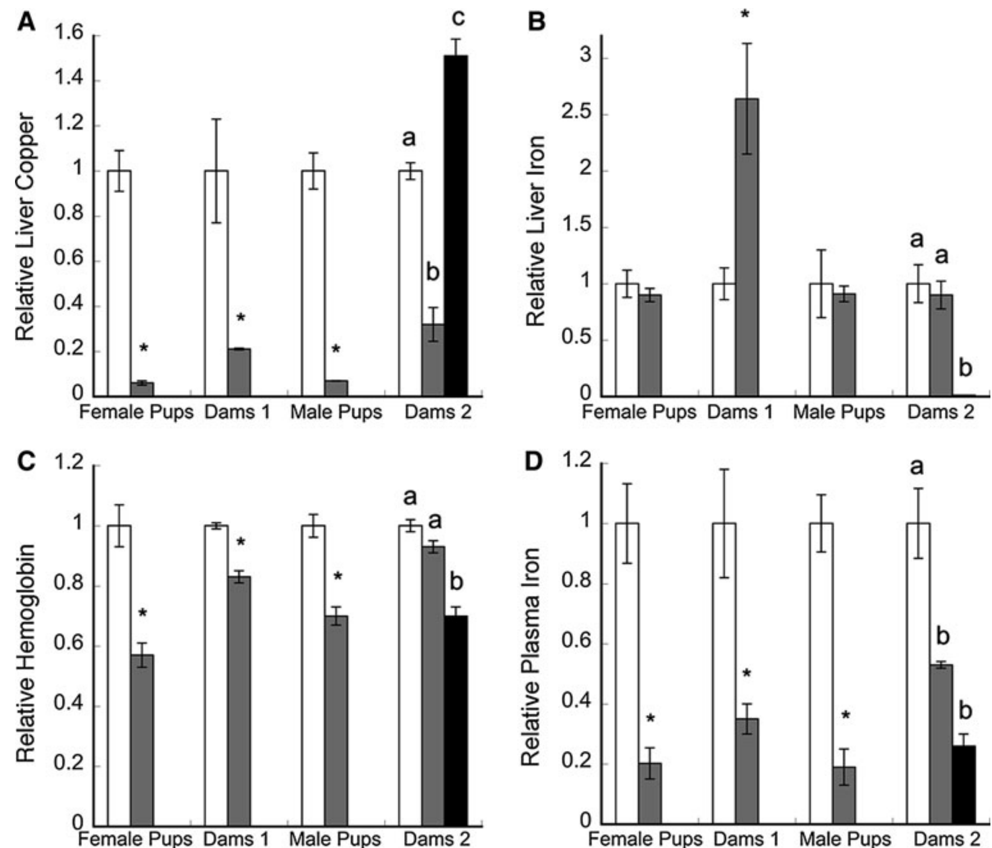

Fig. 1 Relative liver copper (**a**), liver iron (**b**), hemoglobin (**c**), and plasma iron concentrations (**d**) in P26 female pups and Dams1 fed modified AIN-76A diet or P25 male pups and Dams2 fed modified AIN-93G diets. Values are means ± SEM ($n = 4$). Cu-deficient means (*gray fill*) were significantly different than Cu-adequate (*open fill*), $*P < 0.05$ (Student's t test). Dams2 data were analyzed by ANOVA. Means with different letters are significantly different, $P < 0.05$. Iron-deficient *bars* (*solid fill*) are shown

averaging 131 g/L compared to 188 g/L in the CuA Dams2. Plasma iron was measured by flame AAS after hemoglobin removal and was found to be lower in all CuD groups compared to CuA (Fig. 1d). The relative levels of plasma Fe in CuD pups were lower than their respective Dams. Plasma iron level of FeD dams averaged 1.49 μg/mL, about 75 % lower than CuA and 50 % lower than CuD Dams2 (Fig. 1d). Collectively, these data suggest that CuD pups and dams displayed signs characteristic of dietary copper deficiency with hypoferremia.

Impact of copper deficiency on selected hepatic gene expression

Liver mRNA abundance of selected genes was evaluated by qPCR using Gapdh as reference gene. The mean quantification cycle (Cq) for Gapdh abundance for a given experiment was equivalent between CuA, CuD, and FeD liver samples (data not shown). In agreement with their CuD brothers studied earlier (Jenkitkasemwong et al. 2010), liver *Hamp* expression of CuD female pups was nearly non-detectable compared to CuA female liver (Fig. 2a). Similar severe suppression of *Hamp* expression was observed in CuD male Sprague–Dawley pups that consumed the new CuD diet with extra iron (Fig. 2b). Neither CuD female nor male pups had statistically significant alterations in *Fpn* or *Bmp6* mRNA expression (Fig. 2a, b).

In stark contrast to data in pups, the steady state *Hamp* expression in CuD dams for both experiments was not impacted (Fig. 2c, d). As a positive control, a 700-fold reduction in *Hamp* expression in FeD dams liver was detected (Fig. 2d). Similar to pups, neither *Fpn* nor *Bmp6* mRNA levels were statistically impacted by diet in dams. The unexpected finding of no change in *Hamp* expression in CuD dams (Dams1or Dams2) was confirmed several times by re-analysis of fresh cDNA and re-isolation of RNA from an additional sample of frozen liver tissue with equivalent results.

Impact of copper deficiency on selected liver membrane proteins

To evaluate the impact of dietary treatments in pups and dams, liver membranes were prepared, washed free of cytoplasm, and subjected to SDS–PAGE (Fig. 3). Four proteins involved in iron homeostasis and a loading control were evaluated. Densitometry and statistical evaluation of treatment means were conducted (Table 1). The abundance of the loading control SRB1 was quite variable (Fig. 3) despite the appearance of equal loading following Ponceau S staining (not shown). Thus, another loading control actin

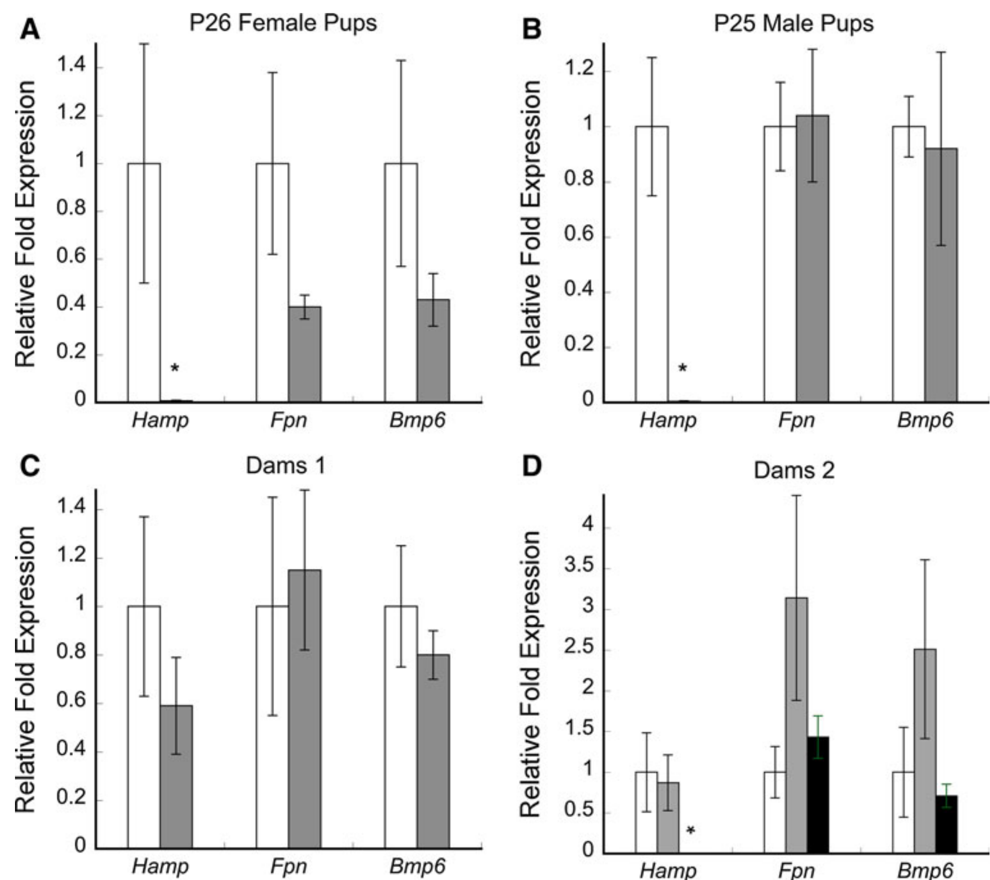

Fig. 2 Relative hepatic gene expression in P26 female rat pups (**a**), P25 male rat pups (**b**) and their respective dams (**c**) and (**d**). Values are means ± SEM ($n = 4$). Cu-deficient means (*gray fill*) and iron-deficient means (*solid fill*) were significantly different than Cu-adequate (*open bars*), $^*P < 0.05$ (Student's t test or ANOVA)

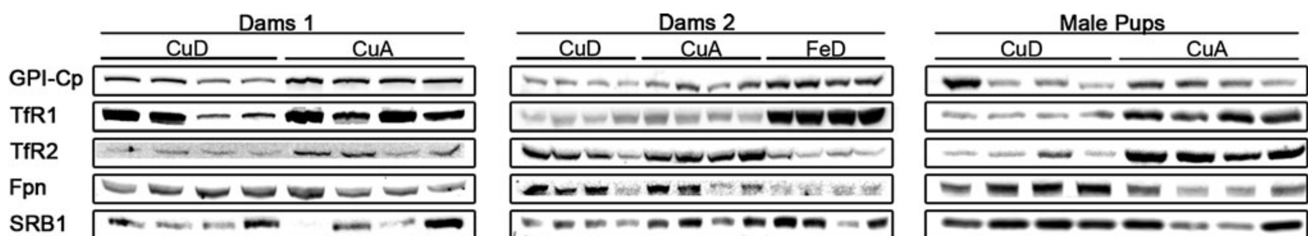

Fig. 3 Hepatic protein levels of GPI-ceruloplasmin, transferrin receptor 1, transferrin receptor 2, ferroportin, and scavenger receptor-binding protein 1 following copper deficiency (CuD) or iron deficiency (FeD) in rat Dams1 fed modified AIN-76A diets, or rat Dams2 and P25 male pups fed modified AIN-93G diets. Immunoblots were performed on 10 % SDS–PAGE gels

was evaluated. Previously, it was reported that actin was not suitable for liver membranes of rat pups (Mostad and Prohaska 2011). That was confirmed again for the P25 male rats. Relative levels of liver actin for the Dams in Expt 1, like SRB1, were not impacted statistically by dietary treatment. Similarly, Dams in Expt. 2 also did not display differences in actin or SRB1 abundance due to diet.

CuD Dams fed CuD AIN-76A diet without copper in drinking water (CuD Dams1) had lower abundance of liver GP1-Cp and TfR2 compared to CuA dams. Levels of Fpn and TfR1 were not different between the two groups. Similar results were obtained for CuD Dams2 fed the new modified AIN-93G diet compared to CuA dams (Fig. 3, Table 1). GP1-Cp and TfR2 abundances were lower, and no significant differences in Fpn or TfR1 were detected. In contrast, the FeD Dams2 had higher liver GP1-Cp and TfR1 expression than CuA dams, but also lower TfR2 abundance. Liver Fpn was not altered statistically in FeD dams though at first glance density appears lower (Fig. 3, Table 1).

Male pups with lower *Hamp* expression had a slightly different profile including a robust augmentation in liver Fpn abundance (Fig. 3, Table 1). CuD male pups also expressed lower levels of GP1-Cp, TfR1, and TfR2 compared to CuA male rats. As a group, all CuD rats had lower GP1-Cp (reflecting copper status) and lower TfR2

Table 1 Selected protein expression in liver and spleen, and spleen non-heme iron (NHI) content following dietary copper or iron deficiency in rats

	Experimental group						
	Dams1		Dams2			Male pups	
	CuD	CuA	CuD	CuA	FeD	CuD	CuA
Liver levels							
GPI-Cp	0.65 ± 0.06*	1.00 ± 0.03	0.64 ± 0.05^{c}	1.00 ± 0.14^{b}	1.52 ± 0.10^{a}	0.40 ± 0.24*	1.00 ± 0.10
TfR1	0.71 ± 0.22	1.00 ± 0.14	0.75 ± 0.13^{b}	1.00 ± 0.10^{b}	4.85 ± 0.18^{a}	0.55 ± 0.02*	1.00 ± 0.05
TfR2	0.80 ± 0.04*	1.00 ± 0.09	0.66 ± 0.09^{b}	1.00 ± 0.11^{a}	0.45 ± 0.07^{b}	0.30 ± 0.05*	1.00 ± 0.12
Fpn	1.06 ± 0.07	1.00 ± 0.15	1.14 ± 0.18^{a}	1.00 ± 0.24^{a}	0.58 ± 0.05^{a}	1.74 ± 0.18*	1.00 ± 0.12
Spleen levels							
GPI-Cp	0.23 ± 0.01*	1.00 ± 0.06	0.12 ± 0.03^{c}	1.00 ± 0.04^{a}	0.78 ± 0.07^{b}	0.51 ± 0.01*	1.00 ± 0.09
TfR1	6.40 ± 0.71*	1.00 ± 0.23	1.44 ± 0.49^{b}	1.00 ± 0.16^{b}	4.04 ± 0.22^{a}	1.94 ± 0.06*	1.00 ± 0.09
Fpn	0.76 ± 0.15	1.00 ± 0.10	0.70 ± 0.05^{b}	1.00 ± 0.13^{b}	1.54 ± 0.16^{a}	1.09 ± 0.11	1.00 ± 0.09
CCS	7.35 ± 0.65*	1.00 ± 0.32	2.61 ± 0.14^{a}	1.00 ± 0.07^{b}	1.41 ± 0.14^{b}	2.88 ± 0.37*	1.00 ± 0.06
NHI, μg/g	462 ± 80.7*	656 ± 33.5	204 ± 39.3^{b}	497 ± 102^{a}	59.8 ± 3.89^{b}	25.0 ± 4.6*	36.2 ± 1.1

Protein expression levels relative to copper-adequate (CuA) rats were determined from densitometric analyses of Western blot data for copper-deficient (CuD) and iron-deficient (FeD) rats (Figs. 3, 4). Means ± SEM ($n = 4$) are shown, * $P < 0.05$ compared to CuA. Dams 2 data were analyzed by ANOVA, means with differing superscripts are different, $P < 0.05$

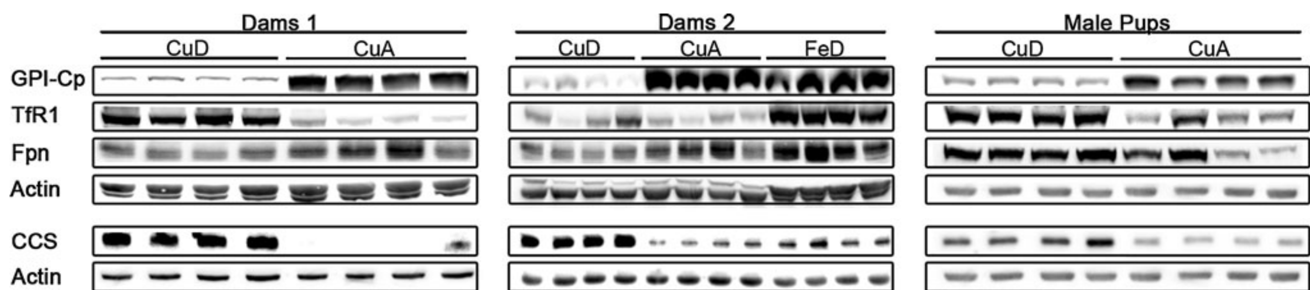

Fig. 4 Splenic protein levels of GPI-ceruloplasmin, transferrin receptor 1, ferroportin, and actin in membranes or copper chaperone for superoxide dismutase and actin in cytosol following copper deficiency (CuD) or iron deficiency (FeD) in rat Dams1 fed modified AIN-76A diets, or rat Dams2 and P25 male pups fed modified AIN-93G diets. Immunoblots were performed on 10 % SDS–PAGE gels for membranes and 15 % gels for cytosol

abundance (reflecting iron status). Fpn was only augmented when *Hamp* was lower (CuD male pups). Interestingly, liver Fpn was not augmented in FeD dams despite low *Hamp* expression. Membranes from livers and spleens of P26 female rats have been evaluated earlier and were not included (Mostad and Prohaska 2011).

Impact of copper deficiency on selected spleen proteins

CuD Dams1 expressed markedly lower spleen GPI-Cp levels than CuA dams (Fig. 4, Table 1). TfR1 levels were augmented greater than fivefold consistent with lower NHI concentration. Spleen Fpn level was equivalent between CuD and CuA dams. Cytosol CCS level was sixfold higher in CuD than CuA spleen from Dams1 confirming severe copper deficiency.

CuD Dams2 also had a marked reduction in spleen GP1-Cp but no change in Fpn level compared to CuA dams (Table 1). Interestingly, CuD Dams2 did not have higher TfR1 levels despite lower NHI level (Fig. 4, Table 1). Cytosol CCS was augmented in CuD Dams2 spleen confirming copper deficiency in this organ. Note CCS was not altered in FeD spleen cytosol of Dams2 (Fig. 4, Table 1).

FeD dams expressed a modest reduction in spleen GP1-Cp, opposite to the finding in liver (Fig. 4, Table 1). As expected, spleen TFR1 was greatly augmented in FeD dams consistent with low NHI (Table 1). Spleen Fpn of FeD Dams2 was 50 % higher than CuA levels.

CuD male pups also displayed a marked reduction in spleen GP1-Cp (Fig. 4, Table 1). TfR1 level was augmented reflecting lower NHI. Spleen Fpn level was not statistically different between CuD and CuA rat pups.

Spleen CCS was augmented in CuD cytosol, as expected, and confirms copper deficiency.

Relationship of plasma iron with liver *Hamp*

The unexpected failure to detect lower *Hamp* expression in both groups of CuD dams prompted further causal analysis. From the evaluation of plasma iron data (Fig. 1), it appeared that CuD rat pup levels were lower than their mothers. Recall, CuD pups had very low liver *Hamp* expression (Fig. 2). Plasma iron level was evaluated versus liver *Hamp* expression in all rat dams to determine whether absolute plasma iron concentration could explain the difference between FeD and CuD dams (Fig. 5). There was clear separation between 8 CuA dams and 8 CuD dams for plasma iron. However, plasma iron level did not correlate with *Hamp* expression, as many CuD dams with *Hamp* expression levels similar to CuA dams had plasma iron levels as low as some FeD dams with extremely low *Hamp* expression (Fig. 5).

Selected plasma protein levels following copper and iron deficiency

Plasma from Dams2 fed modified AIN-93G diets were subjected to SDS–PAGE (Fig. 6). Cp levels were greatly diminished in CuD plasma (0.47 ± 0.07) compared to CuA (1.00 ± 0.03) or FeD (0.98 ± 0.07) dams. Transferrin, a loading control, was not altered by either CuD or FeD treatment. Levels of GDF-15, a putative erythroid cytokine that suppresses hepcidin expression, were not statistically different among groups and importantly not different between CuD and FeD dams with differing hepcidin expression.

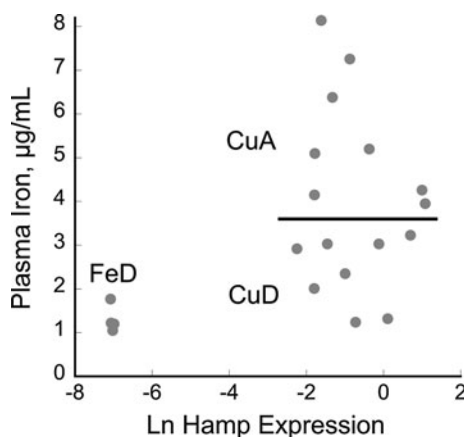

Fig. 5 Correlation between plasma iron concentration and the natural log of liver *Hamp* expression in 20 rat dams maintained on either copper-adequate (CuA), copper-deficient (CuD), or iron-deficient (FeD) treatment. A *bar* separates 8 CuA from 8 CuD values

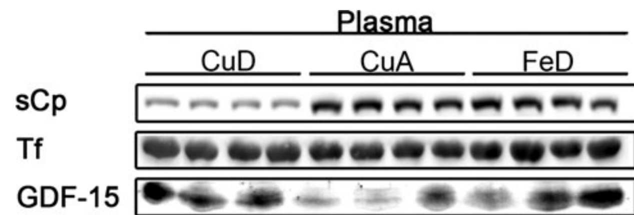

Fig. 6 Plasma protein levels of ceruloplasmin, transferrin, and growth differentiation factor 15 following copper deficiency (CuD) or iron deficiency (FeD) in rat dams fed modified AIN-93G diets. Immunoblots were performed on 10 % SDS–PAGE gels

Discussion

A salient feature of copper deficiency is the development of anemia first documented experimentally in rats in 1928 (Hart et al. 1928). CuD rats develop hypoferremia as displayed in several rat models in the current studies. Iron deficiency also results in hypoferremia and anemia, and it is commonly suggested that anemia of copper deficiency is caused by a secondary (induced) iron deficiency (Chen et al. 2006; Reeves et al. 2005). However, there are exceptions that question this logic. CuD mice often have no changes in plasma iron but are profoundly anemic (Pyatskowit and Prohaska 2008a). Injection of anemic post-weanling CuD rats and mice with iron does not reverse anemia (Prohaska et al. 1985; Reeves and DeMars 2006; Williams et al. 1983). Further, *Cp* −/− mice and humans with aceruloplasminemia exhibit hypoferremia but only mild anemia, as reviewed elsewhere (Prohaska 2011). However, iron delivery to bone marrow may still be partially responsible for anemia.

Why does hypoferremia develop in CuD rodents (Pyatskowit and Prohaska 2008c)? One hypothesis suggests limitation in hephaestin, the MCO of enterocytes (Chen et al. 2006; Reeves et al. 2005). Two studies provided evidence of decreased hephaestin abundance and increased intestinal iron following dietary copper deficiency. However, hephaestin mutants (sex-linked anemia (*sla*)) mice display no anemia as adults nor any reduction in serum iron (Chen et al. 2009). Also, CuD mice with profound anemia do have increased intestinal iron but have normal plasma iron and whole body iron content (Pyatskowit and Prohaska 2008a).

An alternate hypothesis to explain hypoferremia of CuD is limiting function of Cp (Osaki et al. 1966). *Cp* −/− mice and people lacking Cp express severe hypoferremia, as noted above, and also massive hepatic iron overload consistent with the MCO Cp functioning in iron egress from liver. As reported in current studies with Dams 1, CuD rats also have augmented liver iron. This is true for CuD mice on both diets used in the current experiments (Mostad and Prohaska 2011; Pyatskowit and Prohaska 2008a). Puzzling

though is the lack of iron retention in spleen of CuD rats as shown in the current studies as well as previous work (Mostad and Prohaska 2011; Prohaska and Broderius 2012). CuD rats have extremely low levels of plasma Cp and low spleen GPI-Cp. Further, puzzling is that iron levels are actually lower in CuD spleen with low GPI-Cp protein and ferroxidase activity as shown in current data and previously (Mostad and Prohaska 2011). How can iron leave spleen of CuD rats with low GPI-Cp? Perhaps, spleen macrophages express another MCO such as hephaestin that can compensate for the loss of GPI-Cp. Precedence for this exists in brain of *Sla* mice in which GPI-Cp is augmented when hephaestin is not functioning (Schulz et al. 2011). This seems unlikely though as CuD rodents, even if they had splenic hephaestin, should have lower abundance and perhaps non-functional hephaestin based on prior intestinal data (Chen et al. 2006; Reeves et al. 2005).

It is possible that a non-copper-dependent ferroxidase might function. A candidate is APP, the amyloid precursor protein, recently shown to possess ferroxidase activity (Duce et al. 2010). This possibility remains to be tested. It should be noted that spleens of younger *Cp* −/−mice do not display augmented iron, whereas their livers do (Prohaska 2011). Eventually, aged *Cp* −/−mice do demonstrate augmented splenic iron (Harris et al. 1999). Perhaps, APP or some other ferroxidase can explain these splenic iron data. It is also likely that lower splenic iron is the result of lower numbers of circulating erythrocytes following CuD.

A third hypothesis to explain hypoferremia of CuD rats would involve Fpn. Fpn has been shown by others to be essential for life and for iron egress from enterocytes, liver Kupffer cells and hepatocytes, and splenic macrophages (Donovan et al. 2005). Some argue that membrane Fpn expression depends on membrane MCO function such as hephaestin and GP1-Cp (De Domenico et al. 2007; Kono et al. 2010). Published data in male rats and data on CuD rat dams in current experiments demonstrate profound reduction in both liver and spleen GPI-Cp (Mostad and Prohaska 2011). However, current and previously published data suggest that Fpn levels are augmented in CuD liver and sometimes in spleen (Jenkitkasemwong et al. 2010). Certainly, there is no evidence for lower Fpn following CuD in rats or mice (Chen et al. 2006; Chung et al. 2004; Jenkitkasemwong et al. 2010; Mostad and Prohaska 2011; Prohaska and Broderius 2012). Hypoferremic *Cp* −/− mice have augmented intestinal and liver Fpn (Chen et al. 2009). Thus, it seems unlikely that hypoferremia of CuD is due to limiting Fpn.

Fpn expression is extremely complex and controlled partially by transcriptional, posttranscriptional, and posttranslational mechanisms that involve, at least partially, the well-characterized IRE and IRE-BP system (Knutson 2010). Higher iron levels should result in higher Fpn expression. Thus, augmented *Fpn* mRNA and protein in liver of *Cp* −/− mice and intestine of CuD mice follow dogma (Chen et al. 2006, 2009). However, current liver data in CuD rat Dams1, with augmented liver iron, and our previous work in older CuD rat males and CuD mice with augmented iron did not detect higher *Fpn* mRNA.

However, Fpn levels are also dependent on the level of the secreted liver hormone hepcidin (Knutson 2010). In fact, previous work in CuD mice with lower hepcidin and CuD rats with lower hepcidin both reported higher Fpn protein (Chen et al. 2006; Jenkitkasemwong et al. 2010). This suggests that Fpn level is inversely correlated with hepcidin in CuD rodents. In fact, in some CuD mice, liver Fpn was not augmented, likely because hepcidin was not suppressed (Jenkitkasemwong et al. 2010). In the current studies, liver Fpn was augmented in CuD male rat pups whose hepcidin expression was greatly blunted. Somewhat surprising was the failure to detect augmented liver Fpn in FeD dams that also had greatly suppressed levels of hepcidin. Perhaps, in this case, low hepatic iron may be a factor.

Previous and current data suggest there is a strong correlation between augmented Fpn and decreased hepcidin in CuD rats (Jenkitkasemwong et al. 2010). What drives lower hepcidin expression in CuD rats? In CuD male and female rat pups, there is a good correlation between low hepcidin and low plasma iron. This was noted previously as CuD rats with low hepcidin had low plasma iron and Cu mice with normal hepcidin had normal plasma iron; yet, both CuD rats and mice were anemic (Jenkitkasemwong et al. 2010). Thus, in this situation, hepcidin is best correlated with holotransferrin rather than anemia. Elegant studies by others with mutant mice suggest transferrin iron is the major determinant of hepcidin expression (Bartnikas et al. 2011; Wilkins et al. 2006).

One signaling pathway involves Tf, TfR1, HFE, and TfR2 (Gao et al. 2009; Schmidt et al. 2008). Previously, it was suggested that TfR2 levels, lower in CuD rat liver, correlated with lower hepcidin (Jenkitkasemwong et al. 2010). However, data in our current studies detected low TfR2 consistently in all CuD liver samples and in FeD liver; yet, hepcidin was not lower in liver of CuD dams. Further, hepcidin was not suppressed in CuD dams that had plasma iron levels equivalent to FeD dams with major reductions in hepcidin. Thus, it appears there must be some other factor that explains the normal levels of hepcidin in CuD dams even though they express lower plasma iron and in some cases were anemic.

It is known that bone marrow releases cytokines that may block hepcidin expression (Knutson 2010). The nature of these erythroid regulators remains elusive (Bartnikas et al. 2011). One candidate cytokine, GDF-15, does not appear to be that CuD factor based on very limited current

data. Further research will be necessary to evaluate the role of bone marrow in the etiology of anemia following copper deficiency. However, there is evidence for CuD rodents having abnormal bone marrow metabolism (Collins et al. 2010; Karimbakas et al. 1998). Further research will be necessary to identify factors in the CuD dams that allow normal hepcidin expression even when hypotransferrinemia is evident. This may involve factors in hepatic signaling or release of erythropoietic factors from bone marrow. Further, it will be important to determine whether iron uptake is limited in CuD dams due to normal hepcidin expression.

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