Original Research

Glycosylphosphatidylinositol-linked ceruloplasmin is expressed in multiple rodent organs and is lower following dietary copper deficiency

Elise J Mostad and Joseph R Prohaska

Department of Biochemistry and Molecular Biology, University of Minnesota Medical School Duluth, Duluth, MN 55812, USA
Corresponding author: Joseph R Prohaska, Department of Biochemistry and Molecular Biology, University of Minnesota Medical School Duluth, 1035 University Drive, Duluth, MN 55812, USA. Email: jprohask@d.umn.edu

Abstract

Ceruloplasmin (Cp), a multicopper ferroxidase, is expressed as both a secreted (sCp) plasma enzyme from the liver and a membrane-bound glycosylphosphatidylinositol-anchored (GPI-Cp) splice variant protein. Cp is thought to be essential for iron mobilization as selective iron overload occurs in aceruloplasminemia in humans and in Cp null mice. Dietary copper-deficient (CuD) rodents have near total loss of Cp activity, severe loss of Cp protein and develop anemia. Hepatic iron augmentation is often observed, suggesting that loss of Cp function may be correlated with anemia. The impact of CuD treatment on GPI-Cp has not previously been evaluated. Our hypothesis was that CuD rodents would have lower levels of GPI-Cp and this would correlate with higher tissue iron retention. In these studies, GPI-Cp was detected in purified membranes of multiple organs of rats and mice but not *Cp* $-/-$ mice. Immunoreactive Cp protein was released with phosphatidylinositol phospholipase C treatment and expressed ferroxidase activity. Following perinatal and postnatal copper restriction, GPI-Cp was markedly lower in the spleen and modestly lower in the liver of CuD rats and mice, when compared with copper-adequate (CuA) rodents. However, spleen non-heme iron (NHI) was lower in CuD than CuA rats, and not different in CuD mice. Hepatic iron was higher only in CuD mice. Spleen and liver membranes of CuD rats expressed augmented levels of ferroportin, the iron efflux transporter, which may explain lower NHI content in the spleen of CuD rats despite a greater than 50% lower level of the multicopper ferroxidase GPI-Cp. Spleen and liver levels of GPI-Cp mRNA were not impacted in CuD rats, suggesting that turnover rather than biosynthesis may explain the lower steady-state levels of GPI-Cp following dietary copper restriction. Lower GPI-Cp did not correlate with tissue iron retention and thus the role, if any, of Cp in anemia of copper deficiency is unknown.

Keywords: copper deficient, rat, mice, GPI-ceruloplasmin, iron

Experimental Biology and Medicine 2011; **236**: 298–308. DOI: 10.1258/ebm.2010.010256

Introduction

Copper is an essential co-factor for numerous cuproenzymes known to catalyze key biochemical reactions. In general, these cuproenzymes participate in redox chemistry and impact many physiological processes such as pigmentation, catecholamine synthesis, superoxide metabolism, mitochondrial energy production, connective tissue integrity and hematopoiesis.[1] Seminal work in rats described a copper requirement for growth and hemoglobin formation.[2] Later it was shown that iron metabolism requires multicopper oxidases with ferroxidase activity to convert ferrous to ferric iron to facilitate binding to transferrin (Tf), the plasma iron transporter.[3] It is believed that the anemia (low hemoglobin) observed in copper-deficient rats is due to hypoferremia.[4] An interaction between iron and copper was described in humans in the 19th century, but specific details still remain unknown.[5,6] For example, dietary copper deficiency in mice also leads to profound anemia but plasma iron levels are normal.[7] Key in the molecular copper–iron hypothesis is the cuproenzyme ceruloplasmin (Cp).

Cp is an α-2-globulin that binds six atoms of copper, which represents about 95% of the copper found in plasma. This glycoprotein was first characterized in 1947 by Holmberg and Laurell and determined to be synthesized and secreted into plasma by hepatocytes.[8] Cp can oxidize several diamines but is thought to be essential for the ferroxidase pathway of iron mobilization.[3] Consistent with this

hypothesis was the observation that humans lacking *Cp* (aceruloplasminemia) accumulated excess iron in several organs including liver and brain.[9] Experiments with *Cp*−/− mice confirmed these observations and challenged the idea that Cp was involved in copper transport and delivery because liver, spleen, and brain accumulated iron but had normal copper levels.[10] Collectively, these studies strongly supported the Cp ferroxidase hypothesis. Further, when copper is limiting in the diet, Cp activity is markedly lower and liver iron is elevated.[4] However, *Cp* −/− mice, despite tissue-specific iron overload, do not display severe anemia.[10–13] Thus, the mechanism for anemia in copper deficiency remains unknown.

Interestingly, accumulation of iron in the brain in aceruloplasminemia suggested the existence of another form of Cp, since plasma Cp presumably does not cross the blood–brain barrier. Previously Cp was characterized strictly as a secreted protein found in the plasma. It is now known that two isoforms of Cp exist, a secreted form (sCp) and a membrane-bound glycosylphosphatidylinositol-anchored form (GPI-Cp), first described in brain astrocytes.[14] GPI-Cp is generated by alternative splicing of exons 19 and 20, encoding a 1084 amino acid protein, whereas sCp has 1059 amino acids.[15] GPI-Cp has also been reported on the plasma membrane of Sertoli cells and on the surface of leptomeningeal cells in the central nervous system.[16,17] GPI-Cp in brain astrocytes and Sertoli cells of testes suggests a role for GPI-Cp in metabolism of iron at the blood–brain barrier and blood–testis barrier.[18] However, little is known about GPI-Cp in other tissues or its precise role in iron biology and its role, if any, in the mechanism of anemia associated with copper deficiency.

Recent studies confirmed that dietary copper deficiency in rats and mice resulted in a 60–90% decrease in sCp protein abundance.[19] Perhaps this is due to enhanced degradation of unstable apoceruloplasmin.[20] A key objective of the current experiments was to assess the effect of dietary copper deficiency on membrane-bound GPI-Cp. A more thorough investigation of the impact of GPI-Cp on iron retention seemed prudent, as iron imbalance can have very serious physiological consequences besides anemia including diabetes, dementia, oxidative stress and impaired cognitive development.[21] The primary cells involved in iron recycling are splenic macrophages and liver Kupffer cells. *Cp*−/− humans and mice accumulate iron in these macrophages. It is not clear whether reduction of sCp observed in dietary copper deficiency or a putative alteration in GPI-Cp might also impact iron export from spleen and liver.

Current experiments were designed to evaluate GPI-Cp in multiple organs using rodents as models with an emphasis on organs where iron is stored and excreted, including spleen and liver. Impact of dietary copper limitation and related variables (age, gender, species and rat strain) were analyzed. Data demonstrated that GPI-Cp is expressed in multiple organs of rodents, including liver, spleen, kidney, heart and brain. Furthermore, following dietary copper deficiency GPI-Cp expression in spleen, and to a lesser extent in liver, was lower. However, it was also determined that lower GPI-Cp was not correlated with augmented non-heme iron (NHI) levels.

Materials and methods

Animal care and dietary treatments

Sperm-positive Holtzman rats were purchased from Harlan Laboratories Inc (Indianapolis, IN, USA) and weanling male and sperm-positive Sprague Dawley rats and sperm-positive Swiss Webster mice were purchased from Charles River (Wilmington, MA, USA). Rodents were offered either a copper-deficient (CuD) or copper-adequate (CuA) dietary treatment consisting of a CuD-modified AIN-76A diet, or CuD- and CuA-modified AIN-93G diet (Teklad Laboratories, Madison, WI, USA). The modified AIN-76A diet contained 0.32 mg Cu/kg and 47 mg Fe/kg by analysis. All Holtzman dams and offspring were fed this CuD diet. CuA groups drank water supplemented with cupric sulfate, 20 mg Cu/L, and CuD groups drank deionized water. This perinatal Holtzman rat experiment is referred to as Experiment 1 (Exp. 1). Sprague Dawley rats and Swiss Webster mice were fed the modified AIN-93G diet containing 0.46 mg Cu/kg and 78.3 mg Fe/kg (CuD diet) or 8.73 mg Cu/kg and 80.3 mg Fe/kg (CuA diet). Rodents on the modified AIN-93G diets were given deionized water to drink. The Sprague Dawley rat experiment is referred to as Experiment 2 (Exp. 2). A third experiment (Exp. 3) began with weanling male Sprague Dawley rats offered CuA, CuD or an additional modified AIN-93G iron-deficient (FeD) diet, containing 6.2 mg Fe/kg and 7.0 mg Cu/kg. Further details of the diets used and animal husbandry are published elsewhere.[7,22] All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (07:00–19:00 h). All protocols were approved formally by the University of Minnesota Institutional Animal Care and Use Committee.

Holtzman rat dams (Exp. 1) were placed on dietary treatments on embryonic day 7, Sprague Dawley rat dams (Exp. 2) on embryonic day 2 and Swiss Webster mouse dams on embryonic day 17. Offspring in all cases were weaned at postnatal day 20 (P20) and continued on dietary treatment of their respective dams. Exp. 3 male weanling rats began their respective postnatal CuD, CuA or FeD diets at P21.

For Exp. 1, one representative male rat pup from each litter ($n = 4$, CuA and CuD) was anesthetized by ketamine/xylazine injection and killed by cardiac puncture on P25, and on P26 a sister was killed from each litter in the same manner. For Exp. 2, CuA and CuD P25 ($n = 4$ each) male rat pups were killed by decapitation. For Swiss Webster mice, CuA and CuD males ($n = 4$ each) were killed on P27 by decapitation. For Exp. 3, CuD and CuA male rats ($n = 3$ each) were killed at one, two and four weeks after onset of dietary treatments. At P35, three FeD rats were killed. These rats were anesthetized with ketamine/xylazine injection and killed by cardiac puncture. Appropriate tissues from all rodents were harvested, quick frozen in liquid nitrogen and stored at −70°C for analyses. Dr Z L Harris, Vanderbilt University, kindly provided tissues and plasma from C57BL mice of two genotypes, ceruloplasmin null (*Cp*−/−) and wild-type controls (*Cp*+/+).[10]

Biochemical analyses

A 5 μL aliquot of blood from each rodent was mixed with Drabkin's reagent to measure hemoglobin spectrophotometrically.[23] A piece of liver was wet-digested with HNO_3 (Trace

Metal grade; Fisher Scientific, Pittsburgh, PA, USA) and analyzed for total copper and iron content by flame atomic absorption spectroscopy (Model 1100B, Perkin-Elmer, Waltham, MA, USA).[23] Plasma was treated with hot trichloroacetic acid and iron was measured by flame atomic absorption spectroscopy.[24] NHI levels in spleen and liver were determined colorimetrically after acid extraction of tissues.[25] This is necessary, particularly for spleen, where much of the iron is bound to heme from hemoglobin degradation. Protein concentration of membrane and cytosolic extracts was determined using a modified Lowry method.[26] Plasma Cp activity was assessed by measuring plasma diamine oxidase activity using *o*-dianisidine as substrate.[23] Ferroxidase activity was determined on spleen membrane proteins digested with phospholipase C as described below after separation on non-denaturing gels and transfer to polyvinylidene difluoride membranes as described previously for plasma.[19]

Preparation of membrane fractions

Membrane samples for Western blot analysis were prepared by homogenizing frozen rat or mouse spleen, liver, kidney, heart and cerebellum in nine volumes of homogenization buffer containing 10 mmol/L HEPES, 0.1 mol/L KCl, 0.04 mol/L mannitol and 1 mmol/L EDTA (pH 7) with the addition of protease inhibitors (Protease Inhibitor Cocktail; Sigma-Aldrich, St Louis, MO, USA) using a T25 high-speed digital homogenizer (IKA Works, Inc, Wilmington, NC, USA). Homogenates were centrifuged at 2000*g* for 10 min at 4°C and supernatant fractions were centrifuged again at 100,000*g* for 30 min at 4°C. The supernatant from this spin was saved as the cytosolic extract and the pellet was washed with homogenization buffer and recentrifuged at 100,000*g* for 30 min at 4°C, discarding the supernatant. Pellets were resuspended in 10 mmol/L potassium phosphate buffer (pH 6.8) containing 4% SDS and 0.2% Triton X-100 and are referred to as membrane extracts.

PI-PLC treatment

Selected membrane pellets were suspended in 100 μL of 50 mmol/L Tris-HCl (pH 7.4) containing 10 mmol/L EDTA. These solutions were treated with either 0.7 units of specific phopholipase C (PI-PLC) (P5542, Sigma-Aldrich) or 0.1 μg of bovine serum albumin (BSA) as negative control (Sigma-Aldrich).[27] Pellet suspensions were incubated at 37°C for one hour with mixing every 15 min. Extracts were spun at 100,000*g* for 15 min and the supernatant was removed and saved as the released protein fraction. The treated membrane pellet was washed, the supernatant discarded and the pellet was resuspended in membrane extraction buffer. Aliquots of the resuspended treated membrane fractions and released extracts were used for protein determination and Western blots.

Analysis of mRNA expression in rat tissues

Total RNA was isolated chemically from fast frozen rat tissues using TRI reagent (Ambion, Austin, TX, USA) following the manufacturer recommendations, including suggested optional steps. The concentration of purified RNA was measured with a Nanodrop spectrophotometer and integrity was evaluated by denaturing agarose gel electrophoresis. DNAse treatment used a DNA-free kit (Ambion) and cDNA was synthesized with Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). Tissue cDNA was amplified and quantified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using Rotor-Gene SYBR Green PCR Kit (Qiagen) and Corbett RotorGene RG-3000 to determine C_T values. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Agarose gel analysis showed each primer pair to yield a single band of the expected size. Each PCR reaction contained cDNA synthesized from 15 ng total RNA in a 12.5 μL total reaction volume. sCp and GPI-Cp primers were those used previously for rat kidney experiments.[28] C_T values were confirmed with another set of specific primers for sCp and GPI-Cp used for mouse retina (data not shown).[29] Hephaestin primers designed for qRT-PCR based on NM_133304 were: reverse: 5′-TCC CAG CTT CTG TCA GGG CAA TAA-3′ and forward: 5′-TGT TCC AGT CAT CAA GAC AGC CCA-3′. Amplicon size was 107 bp. Forward and reverse primers used for GAPDH analysis produced a 87-bp product.[30]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Membrane proteins were mixed with SDS Laemmli sample buffer without boiling and electrophoretically fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. Cytosolic samples were treated similarly, but first boiled for five minutes and fractionated on 15% gels. Separated proteins were transferred to a Protran BA 83 nitrocellulose membrane (Whatman) and treated as described previously to detect the appropriate antigens.[19] Immunoreactivity was visualized using SuperSignal chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA). Chemiluminescence detection and densitometry were carried out using the FluorChem™ system (Alpha Innotech, San Leandro, CA, USA).

Antibodies

The following antibodies were purchased commercially and used at appropriate dilutions: anti-mouse actin (MAB1501, Millipore, Bedford, MA, USA); mouse anti-rabbit sodium potassium ATPase (Na/K ATPase) (ab7671, Abcam, Cambridge, MA, USA); mouse anti-human transferrin receptor (TfR) (13–6800, Zymed Laboratories, South San Francisco, CA, USA); goat anti-human Cp (C 0911, Sigma-Aldrich); and goat anti-rabbit lactate dehydrogenase (LDH) (AB1222, Millipore). Affinity-purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and rabbit anti-rat/mouse ferroportin (Fpn) were characterized previously.[31,32]

Statistical analysis

Means ± SEM were calculated. Student's unpaired two-tailed *t*-test was used for the comparison of data between two

dietary treatments ($\alpha = 0.05$) and *F*-test was used to determine the variance equality. Data were processed using Microsoft Excel™. Experiments comparing CuD, FeD and CuA data were evaluated by one-way analysis of variance and Tukey's test after variance equality was evaluated by Bartlett's test (KaleidaGraph, Synergy Software, Reading, PA, USA).

Results

Characterization of membrane-anchored Cp

Membranes were isolated from multiple organs of two CuA mouse dams and detergent-extracted proteins were separated by SDS-PAGE and Cp was detected by immunoblotting. When probed with polyclonal anti-human Cp antibody, a robust band in both mice was detected with an apparent molecular weight of 130 kDa for spleen, liver, kidney and heart. Cp protein was more abundant in spleen and kidney than in liver and heart (Figure 1a). Mobility of the Cp in membrane extracts was faster than plasma Cp, used as a control, indicating unique features. The immunoblot was reprobed for LDH and no bands were detected, suggesting the membrane preparations were not contaminated with plasma or cytoplasm (data not shown). No immunoreactive Cp was detected in isolated rat erythrocyte ghosts (data not shown), suggesting that splenic Cp was not due to red cell membrane contamination. Further studies on a pair of four-month-old male mice, *Cp*+/+ or *Cp*−/−, were conducted to confirm that immunoreactivity was indeed Cp. Membranes from multiple organs of these mice were extracted and immunoreactivity was not detected in *Cp*−/− mouse membranes or plasma (Figure 1b). Cerebellum and heart tissues have less dense membrane Cp bands and spleen the most dense. Actin (44 kDa) was used as a loading control for this blot and all other Cp blots. However, robust actin bands were not detected for young male rat liver membrane preparations. Actin density was tissue-specific, but not impacted by Cp genotype (Figure 1b).

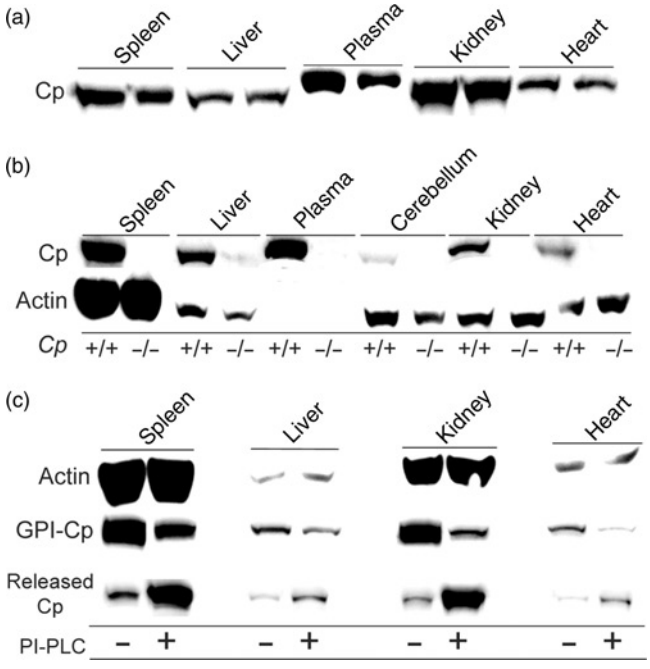

Figure 1 Characterization of rodent GPI-Cp. (a) Membranes from multiple organ samples from two CuA mouse dams were isolated and membrane extracts (50 μg/lane) were separated by 10% SDS-PAGE. Cp (130 kDa) abundance was analyzed by immunoblot. (b) Multiple organs from two male mice, *Cp*+/+ and *Cp*−/−, were evaluated as described above. Actin (44 kDa) was used as a loading control. Mouse plasma 1 μL was also processed for each blot. (c) Exp. 2 P25 male rat multiple membrane samples were incubated with either 0.7 units of PI-PLC (+) or negative control (−) for one hour. The treated membrane fractions (GPI-Cp) and the released protein fractions (Released Cp) were subjected to immunoblot analysis of Cp. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PI-PLC, phopholipase C

Next, additional experiments were carried out with rat membranes to extend mouse data and to determine whether the detected Cp protein was GPI-anchored. Spleen, liver, kidney and heart membrane pellets from a P25 CuA male rat, Exp. 2, were treated with either PI-PLC or a negative control protein (BSA) (Figure 1c). Membrane fractions were processed to separate the treated membrane fraction pellet from released supernatant extracts. Resuspended PI-PLC-treated membrane fractions displayed less remaining immunoreactive Cp than BSA-treated fractions (Figure 1c). GPI-Cp released from the membrane fraction was recovered in the extract of these four rat organs and suggests that immunoreacive Cp was GPI-anchored in these membranes. Released extracts from spleen and kidney possessed ferroxidase activity in a gel/membrane assay (data not shown).[19] Actin abundance was not impacted by PI-PLC treatment.

Relative mRNA expression levels for three multicopper oxidases including both the secreted form (sCP) and the GPI-anchored form of Cp were determined in multiple organs of a representative P27 male CuA rat by qRT-PCR. Primers, designed by others, specific to each of the forms of Cp, were used (Supplemental Table 1). Secreted Cp, as expected, was expressed highest in the liver. Importantly, GPI-Cp was expressed in all of the organs tested and had its highest expression in the kidney, consistent with immunoblot data. Hephaestin, another multicopper oxidase, displayed highest expression in enterocytes, consistent with previous data.[33]

Confirmation of copper deficiency

One mouse model and three rat models of dietary copper deficiency were examined to determine whether alterations observed in sCp levels might occur with GPI-Cp.[19] The perinatal models of Cu deficiency (mouse experiment and rat Exps. 1 and 2) produced CuD pups with features consistent with severe deficiency. CuD pups were smaller in size than CuA rodents (Table 1). Hemoglobin levels for these CuD rodents were significantly lower than their CuA counterparts, ranging between 57% and 71% of CuA values. Liver copper levels were markedly lower in all CuD rodents. Liver iron was 104% higher in CuD than in CuA mice. For CuD rats, the liver iron data were less clear. In fact, the reason that CuA rats in Exp. 1 had lower liver iron than CuD rats may be due to lower dietary iron rather than copper deficiency, since male P25 CuA pups in Exp. 2 had iron levels greater than CuA pups in Exp. 1. Serum iron

Table 1 Characteristics of rats and mice following copper deficiency

Characteristics						
Group	**Diet**	**BW (g)**	**Hb (g/L)**	**L-Cu (μg/g)**	**L-Fe (μg/g)**	**S-Fe (μg/mL)**
Exp. 1 Male rats	CuA	78.6 ± 3.1	108 ± 3.6	6.86 ± 0.81	32.3 ± 2.35	4.66 ± 0.68
	CuD	59.1 ± 8.4*	69.4 ± 3.0*	0.40 ± 0.11*	60.1 ± 6.2*	0.78 ± 0.12*
Exp. 1 Female rats	CuA	71.5 ± 4.1	124 ± 8.3	6.86 ± 0.64	76.2 ± 8.9	nd
	CuD	53.2 ± 3.2*	70.5 ± 6.1*	0.42 ± 0.10*	68.3 ± 5.3	nd
Exp. 2 Male rats	CuA	73.2 ± 3.8	128 ± 6.2	8.80 ± 0.69	60.3 ± 18.0	5.34 ± 0.57
	CuD	62.8 ± 5.9	90.6 ± 3.8*	0.63 ± 0.02*	55.1 ± 4.2	1.02 ± 0.32*
Male mice	CuA	22.7 ± 1.4	138 ± 3.4	4.25 ± 0.16	113 ± 17	3.53 ± 0.10
	CuD	15.2 ± 0.7*	82.3 ± 8.3*	1.85 ± 0.27*	230 ± 43*	4.24 ± 0.66

Values are means ± SEM ($n = 3$ or 4) for P25-P27 rats and mice. Body weight (BW), hemoglobin (Hb), liver copper (L-Cu), liver iron (L-Fe) and serum iron (S-Fe) were determined in copper-adequate (CuA) or copper-deficient (CuD) rodents as described in Materials and methods. An asterik indicates that CuD means were different than CuA, $P < 0.05$ (Student's *t*-test)

concentration was significantly lower in CuD male rats but not altered in CuD mice. Plasma diamine oxidase activity of all CuD rodents was non-detectable (data not shown). Collectively, CuD rats and mice displayed signs characteristic of CuD rodents noted previously.[7]

Copper chaperone for superoxide dismutase (CCS) immunoblots were prepared using the cytosolic fraction of the membrane preparations to evaluate the copper status of spleen and cerebellar tissue as limited sample size prevented metal determination (Figure 2). Previous work indicated that tissue CCS levels were higher following copper deficiency.[32] CCS bands (37 kDa) from CuD samples were markedly denser than corresponding bands from CuA samples, verifying copper deficiency in the CuD rodents. Actin density confirmed equal loading for each immunoblot and was not impacted by copper status.

Impact of copper deficiency on GPI-Cp in young rats

Liver and spleen were organs of particular interest to evaluate regarding copper status and GPI-Cp expression. Spleen membranes from CuD and CuA male rat pups from Exp. 1 (Figure 3a) and Exp. 2 (Figures 3b, c and d) were examined. Immunoblots from both experiments showed a significant decrease in GPI-Cp abundance in CuD rats compared with CuA controls, 63% and 49% lower, respectively (Figures 3a and b). These very similar results were obtained using two different CuD diets and two different rat strains. Interestingly, NHI was significantly lower in spleen of CuD rats to a similar degree in both experiments, suggesting a disconnect between the NHI and GPI-Cp function. Spleen membranes from another set of littermates in Exp. 2 were used to study the release of GPI-Cp and were also probed for GPI-Cp, actin and Fpn. Spleens of these CuD rats had lower GPI-Cp, 64%, but higher Fpn, 37%, than CuA rats (Figure 3c). The PI-PLC released material was detected with anti-Cp antibody following separation on a non-denaturing gel. Ferroxidase activity could be detected in the CuA but not CuD samples, confirming both lower protein abundance and impaired activity of GPI-Cp following copper deficiency (Figure 3d).

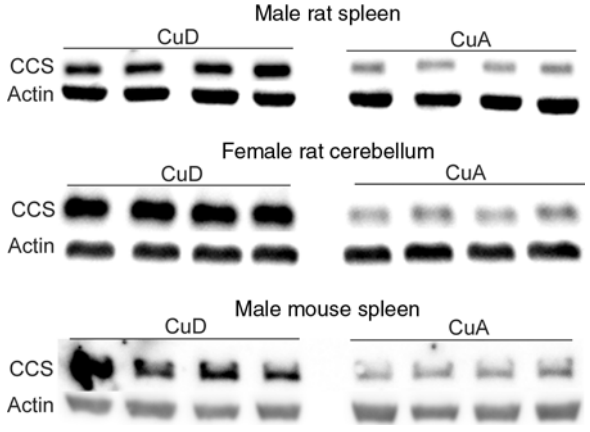

Figure 2 Verification of copper status in multiple rodent tissues by CCS abundance. Cytosolic fractions (20 μg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols. Rodent samples (Exp. 2 P25 male rats, Exp. 1 P26 female rats and P27 male mice) were compared for abundance of CCS (37 kDa) to evaluate copper status. Blots were reprobed for actin. CCS, copper chaperone for superoxide dismutase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CuA, copper-adequate; CuD, copper-deficient

The response of GPI-Cp to dietary copper deficiency was also examined in male rat liver for both Exps. 1 and 2 (Figure 4). Results were not as definitive as those for the spleen. Abundance of GPI-Cp tended to be lower in CuD rats in both experiments, but was not statistically different. Sodium–potassium ATPase was used as a loading control for the liver membrane samples but it was apparent that proper loading could not be determined measuring this protein. Tubulin also appeared to be impacted by diet (data not shown). Recall that actin blotting also was not acceptable for young male rat liver membranes. Ponceau S staining suggested equal loading of both immunoblots (data not shown). Membrane extracts were also probed for Fpn from livers of rats in Exp. 2 (Figure 4b). Robust enhancement of Fpn expression, 74%, was detected in CuD compared with CuA rats, $P < 0.05$.

Membrane preparations from P26 Exp. 1 female rats were evaluated to compare with male rat data (Figure 5). GPI-Cp density was 45% lower in the CuD female spleen. Liver GPI-Cp membrane extracts followed the same trend as the young male rats, appearing lower but not statistically significant. Cerebellum samples from P26 females showed no difference in GPI-Cp expression between CuD and CuA rats. CCS results verified the extent of copper deficiency in the cerebella of CuD female rats (Figure 2). Actin was

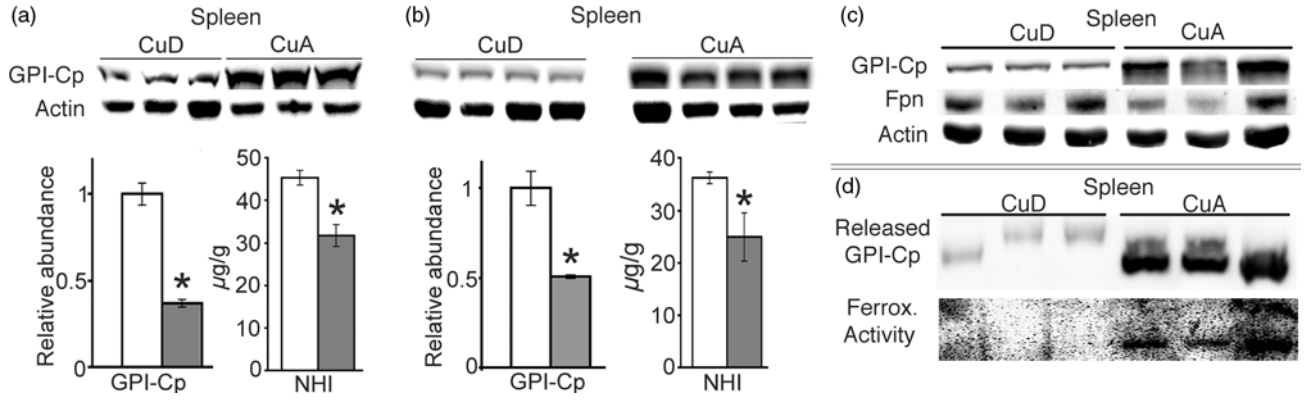

Figure 3 Impact of copper status on spleen GPI-Cp in male rats. Spleen membrane extracts (50 μg/lane) from (a) CuD and CuA (Exp. 1 P25) and (b) CuD and CuA (Exp. 2 P25) were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for actin. NHI was determined spectrophotometrically. White bars represent CuA rats, gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$. (c) Spleen membrane extracts (50 μg/lane) from additional male rats in Exp. 2 were subjected to Western immunoblot protocols for GPI-Cp, Fpn and actin. Abundance of GPI-Cp was significantly lower and Fpn was higher in CuD than CuA samples, $P < 0.05$. (d) Spleen membranes from the same six rats were treated with PI-PLC and the released protein was fractionated on non-denaturing gels, transferred to PVDF membranes and assayed for ferroxidase activity. Membrane was then probed for Cp protein abundance. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient; NHI, non-heme iron; PI-PLC, phopholipase C; Fpn, ferroportin; PVDF, polyvinylidene difluoride

used as a loading control for all female membrane preparations and was not impacted by copper status.

Impacts of perinatal copper deficiency on GPI-Cp in young mice

Spleens and livers of P27 CuD and CuA male Swiss Webster mice were evaluated for GPI-Cp following perinatal copper deficiency to compare with rat data. Spleen GPI-Cp mouse expression confirmed data in rats; levels in CuD mice were 44% lower than CuA (Figure 6a). Spleen NHI levels were not significantly affected by copper deficiency in mice. Like CuD rat liver, CuD mouse liver tended to have a modest reduction in GPI-Cp, though not significant (Figure 6b). However, an overall statistical evaluation of liver immunoblot densities for GPI-Cp of all CuD and CuA rodent comparisons resulted in a robust difference ($P < 0.01$) between the treatment groups, consistent with spleen data. Actin was an effective loading control in mouse tissues and demonstrated equal loading.

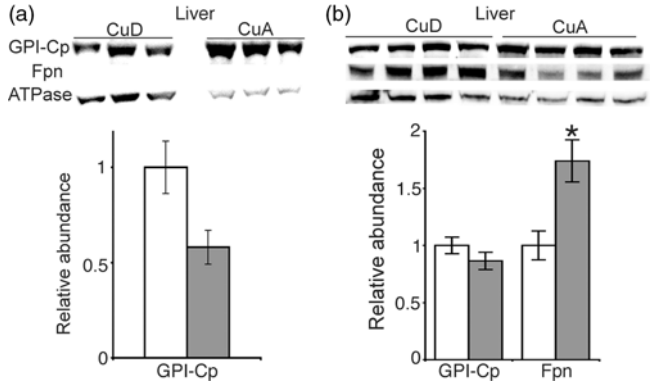

Figure 4 Impact of copper status on liver GPI-Cp in male rats. Liver membrane extracts (50 μg/lane) from (a) CuD and CuA (Exp. 1 P25) and (b) CuD and CuA (Exp. 2 P25) were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for Na/K ATPase. Membranes from Exp. 2 were also probed for Fpn. White bars represent CuA rats, gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient; Fpn, ferroportin

Postnatal copper deficiency

The preceding studies were performed on rodents with severe copper deficiency following perinatal treatments. CuD rodents had frank anemia and reduced body weights (Table 1). Exp. 3 was conducted with older postweanling rats to determine how quickly a change in GPI-Cp might occur following a CuD or FeD diet. Following four weeks of treatment, CuD rats developed modest signs of copper deficiency, mild anemia, but no differences in body weight compared with the CuA rats (data not shown). Rats were sampled one, two and four weeks after diet treatment. Spleen and liver membrane extracts were evaluated at P35 to include data on FeD rats (Figure 7a). Spleen GPI-Cp abundance was markedly lower (65%) in CuD samples following just two weeks of treatment, confirming and extending data in the perinatal rat and mouse models. In liver, but not in spleen, GPI-Cp abundance was actually higher in FeD rats, $P < 0.05$. Iron regulation of TfR appears to be proper only in liver as a robust enhancement is evident in the FeD samples, consistent with the very low NHI levels (Figure 7a). Interestingly, in spleen, despite low levels of NHI in both CuD and FeD rats, TfR expression was not augmented. Reduction of spleen GPI-Cp abundance in CuD rats to less than 50% of levels in CuA rats occurred less than one week after diet-induced copper deficiency (Figure 7b). After four weeks on the CuD diet, spleen GPI-Cp of the CuD rats was less than 20% of the CuA values. Liver copper content displayed a similar response as spleen GPI-Cp results. After the first week on the CuD diet, liver copper

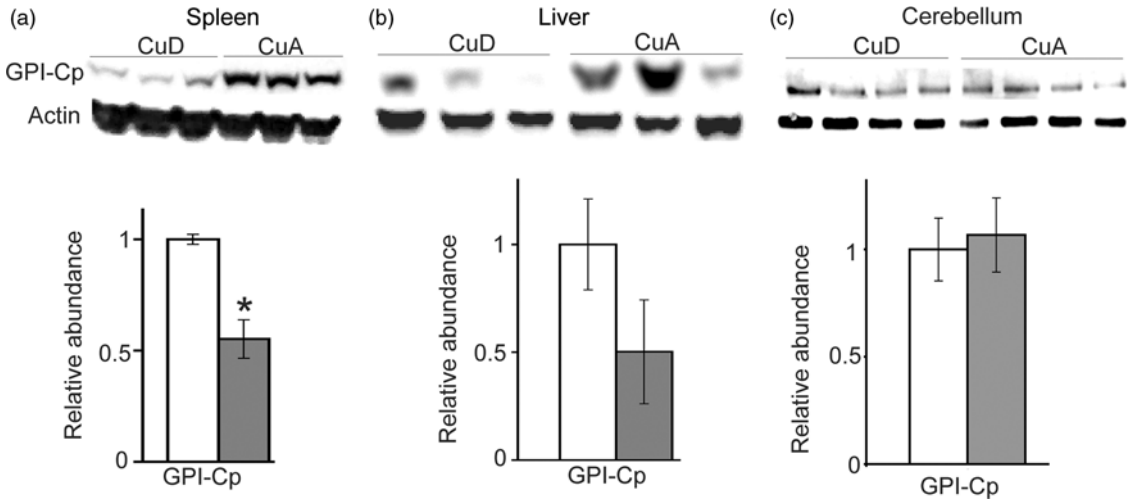

Figure 5 Impact of copper status on GPI-Cp in female rats. (a) Spleen, (b) liver and (c) cerebellum membrane extracts from CuD and CuA female (Exp. 1 P26) rats were subjected to Western immunoblot protocols for GPI-Cp abundance. All blots were reprobed for actin. White bars represent CuA rats; gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient

levels dropped to less than 60% of the CuA values and after four weeks, liver copper levels were less than 40% of CuA values. Although liver GPI-Cp for P35 CuD rats was similar to CuA levels (Figure 7a), levels for CuD rats at P49 were 53% lower than CuA values, $P < 0.05$ (Figure 8a). The CuD state of both spleen and liver from CuD rats was verified when cytoplasmic CCS levels were evaluated (Figures 7c and 8b). Cp diamine oxidase activity was not detectable in the CuD rat plasma. Cp activity in the P35 FeD rats, 78.2 ± 7.3 units/L, was not significantly different than CuA rats, 90.2 ± 7.5, $P > 0.05$.

At P49, both liver and spleen GPI-Cp protein abundance was significantly lower in CuD samples (Figure 8a). RNA isolated from a sample of the same tissues was evaluated for mRNA content of GPI-Cp and GAPDH as a control gene. Relative mRNA expression of spleen and liver GPI-Cp was not altered by CuD (Figure 8a).

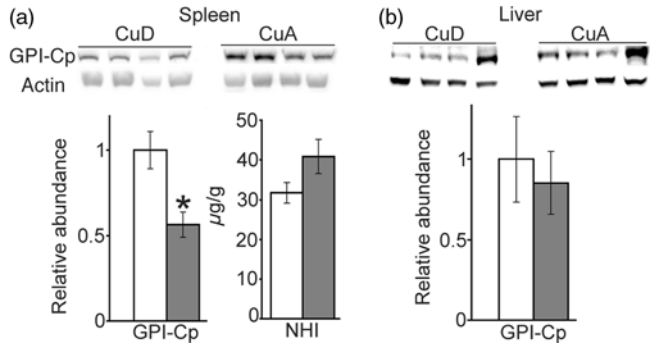

Figure 6 Impact of copper status on GPI-Cp in male mice. (a) Spleen and (b) liver membrane extracts from CuD and CuA P27 male mice were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for actin. NHI was determined spectrophotometrically. White bars represent CuA rats; gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient; NHI, non-heme iron

Discussion

Numerous investigations have probed factors that impact expression and function of secreted plasma ceruloplasmin (sCp). Results in the current studies with rat and mouse tissues clearly demonstrate the presence of both mRNA and protein for the membrane-bound form of this multicopper oxidase (GPI-Cp). Previous studies detected Cp mRNA in numerous tissues, including lung, spleen, testes, placenta and yolk sack, in addition to robust expression in liver.[18] Our data confirm and extend those observations, and those made by others, who have reported the presence of the GPI-anchored form of Cp in selected cells. In particular, GPI-Cp has been reported in brain astrocytes and leptomeningeal cells, murine retina, rat Sertoli cells, rat kidney parietal epithelial cells and human peripheral lymphocytes, especially natural killer cells.[14,16,28,29,34] It was speculated that the presence of GPI-Cp in brain, kidney and retina corresponds to blood–tissue barrier trafficking of iron. Data from the current investigation suggests that GPI-Cp is present in tissues known to have a role in iron trafficking, liver and spleen. It is well known that the iron exporter Fpn is critical for iron efflux. It was clearly demonstrated that the *Fpn* knockout mouse accumulated iron in the absorptive enterocyte, splenic macrophage and liver Kupffer cell.[35] It is proposed that GPI-Cp helps facilitate iron efflux in macrophage-like cells of the liver and kidney in conjunction with Fpn in a parallel manner to the multicopper oxidase hephaestin, a Cp homologue, found predominantly in enterocytes.[5] Mutations in hephaestin, documented in the *sla* mouse, lead to iron retention in enterocytes presumably due to a block in intestinal iron efflux.[33]

Liver is a major homeostatic organ for copper biology. One important function is to synthesize and secrete sCp. It is well known that the liver of CuD rats and mice accumulate iron predominantly either as ferritin or hemosiderin.[36] As mentioned previously, aceruloplasminemia leads to a lack of iron recycling through Kupffer cells because of a decrease

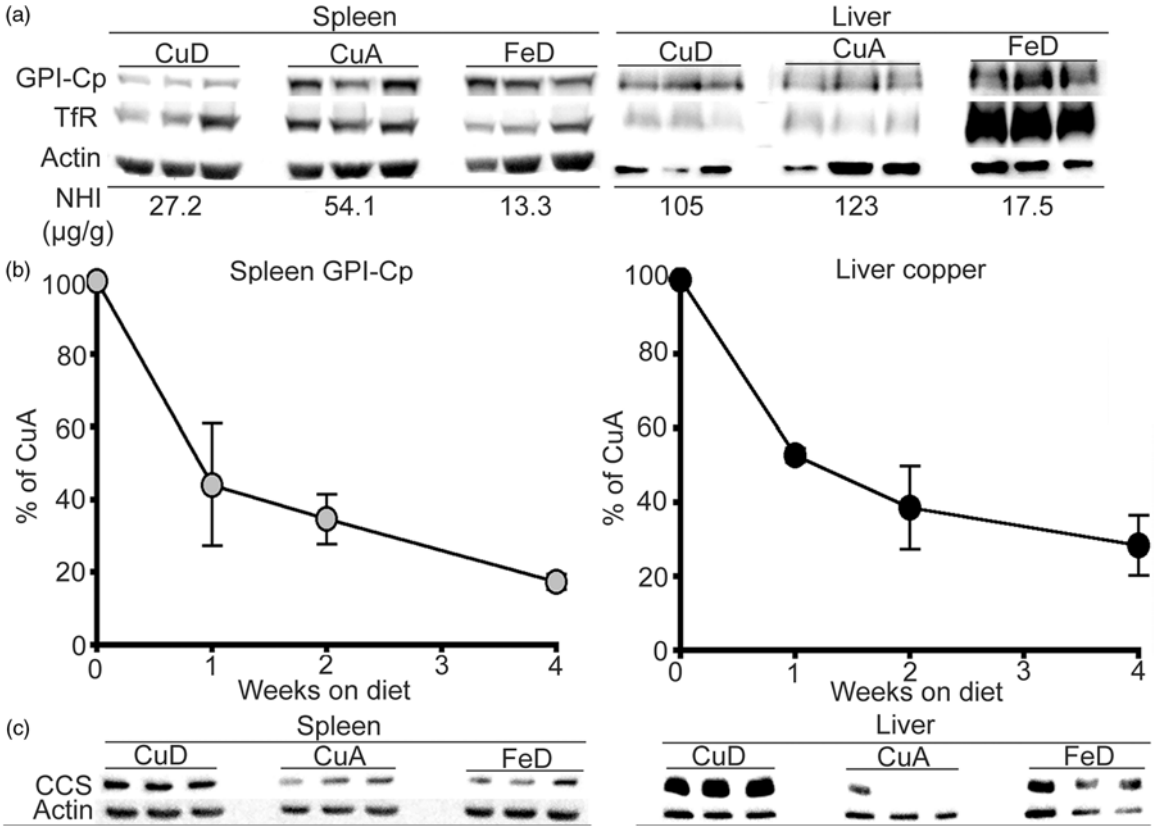

Figure 7 Impact of postnatal copper or iron deficiency in male rats on GPI-Cp abundance. (a) Spleen and liver membrane extracts from CuD, CuA or FeD (Exp. 3 P35) male rats were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were stripped and reprobed for TfR (95 kDa) and actin. NHI was determined spectrophotometrically. Mean values for spleen and liver are listed. (b) Relative changes in spleen GPI-Cp and liver copper concentration in CuD rats, compared with CuA, following dietary copper deficiency. Standard error (SE) bars are shown at each time point. CuD means were significantly lower than CuA for all time points in both comparisons, $P < 0.01$. (c) Spleen and liver cytosolic fractions (20 μg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols. CuD, CuA and FeD samples were compared for abundance of CCS to verify copper status. Both blots were reprobed for actin. CCS was augmented in CuD extracts in both organs, but not altered in FeD samples, $P < 0.05$. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient; FeD, iron-deficient; NHI, non-heme iron; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CCS, copper chaperone for superoxide dismutase

in ferroxidase function. Recent data from our lab on both CuD rats and mice confirmed earlier work that the predominant form of Cp in the plasma following copper deficiency is the apo form of the protein lacking ferroxidase activity.[19] Earlier work in CuD rats and in mice with a deletion of the copper transporter Ctr1 in intestine had convincingly shown that apo-Cp was the major plasma form following copper limitation.[20,37] Limitation in sCp function may restrict iron efflux. Data in the current experiments were collected to test the hypothesis that iron retention in the liver may also be due to a decrease in GPI-Cp. Lower GPI-Cp abundance in hepatic tissue of both CuD rats and mice is reported, albeit only modestly impacted. Perhaps though, the GPI-Cp that is detected does not possess full ferroxidase activity as suggested by the current data on spleen membrane GPI-Cp from CuD rats. The apparent decrease in hepatic GPI-Cp abundance is likely not related to the iron content of the hepatocytes. Two perinatal rat experiments were conducted and there was a difference in liver iron concentration in only one; yet, both showed a similar reduction of liver GPI-Cp abundance.

In the current experiments, liver iron was only augmented in CuD mice. This was verified by measurement of total iron and reflected in the decreased expression of TfR in the CuD mouse liver (not shown). Diets used in Exps. 2 and 3 contained more iron and may explain why there was no apparent difference in liver iron in those rats. It is possible that the iron in CuD rat liver is trapped in hemosiderin and not available for rapid export.[36] Although these liver iron data are somewhat confusing, they confirm recent interesting observations of differing responses to copper deficiency between rats and mice in regard to iron homeostasis. For example, CuD rats experience hypoferremia whereas CuD mice do not.[7]

Splenic macrophages are key in iron recycling that occurs when phagocytized erythrocytes are processed by the spleen.[5] Current data suggest that spleen contains robust expression of GPI-Cp and that abundance is dependent on adequate copper. Significant (~50%) reductions in the expression of GPI-Cp in spleen were observed in several different CuD rat and mouse models ranging from severe to modest copper deficiency. In fact, significant reduction in spleen GPI-Cp abundance was detected after only seven days on CuD treatment. Although the cell-specific expression was not confirmed in these studies, we presume that GPI-Cp is macrophage associated. Previous studies suggested that mouse macrophage bone marrow cells express GPI-anchored Cp.[38] Recent work in CuD rats

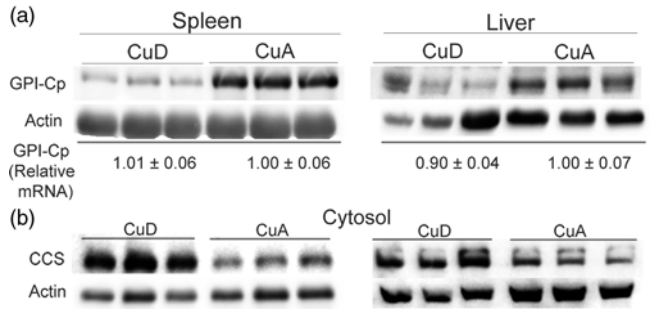

Figure 8 Impact of postnatal copper deficiency in male rats on GPI-Cp protein and mRNA abundance. (a) Spleen and liver membrane extracts from CuD and CuA (Exp. 3 P49) male rats were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were stripped and reprobed for actin. Relative spleen and liver GPI-Cp mRNA levels in CuD and CuA rats was determined by qRT-PCR. Mean C_T values were determined for each rat and organ for both GPI-Cp and GAPDH. Delta C_T values were calculated and the mean CuA value was assigned a value of 1.0; all individual values were then calculated and means were compared. There were no significant differences between CuA and CuD for either spleen or liver (mean $\pm$ SEM), $P > 0.05$. (b) Spleen and liver cytosolic fractions (20 μg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols. CuD and CuA samples were compared for abundance of CCS to evaluate copper status. Both blots were reprobed for actin. CCS was augmented in CuD extracts in both organs, $P < 0.05$. Cp, ceruloplasmin; GPI, glycosylphosphatidylinositol; CuA, copper-adequate; CuD, copper-deficient; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CCS, copper chaperone for superoxide dismutase; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

and mice detected residual levels of sCp in plasma but was unable to detect ferroxidase activity.[19] It is possible that the residual GPI-Cp in spleen of CuD rodents has ferroxidase activity but our gel assay detected none. Interestingly, in the current studies, with diminished levels of GPI-Cp in spleen, there was no concomitant retention of iron. In fact, lower NHI was detected in all CuD splenic samples from rats. There was no difference in NHI in spleens of CuD mice. Once again, it is not known which cells contain the majority of NHI in spleen, but it is likely to be splenic macrophages, the same cells which express GPI-Cp. Perhaps this disconnect in CuD rats between the abundance of GPI-Cp, a relative reduction, and NHI, a relative reduction, can be explained by the recent observation that Fpn abundance is higher in spleen of CuD Holtzman rats.[31] That same study showed that mouse Fpn abundance was not changed following copper deficiency. Our current data detected higher Fpn expression in both CuD liver and spleen in Sprague Dawley rats using a different diet, modified AIN-93G, that eliminated hepatic iron differences.

Higher expression of splenic and hepatic Fpn was observed concurrently with decreased expression (and presumably activity) of the multicopper oxidases sCp and GPI-Cp (current data).[19,31] Others based on *in vitro* experiments suggest that Fpn abundance depends on active multicopper oxidases, so as to prevent internalization and degradation via the proteasome.[38] That theory predicted lower Fpn abundance in CuD tissues. An alternate hypothesis suggests that higher Fpn in the spleen and liver of CuD rats was due to near total loss of hepcidin expression.[31] Hepcidin binding to Fpn is also a mechanism to control the steady-state levels of Fpn protein and iron flux.[39] In support of the disconnect between lower multicopper oxidase and higher Fpn expression is the work with CuD mice from others that showed a higher expression of enterocyte Fpn concomitant with a decrease in hephaestin, another multicopper oxidase, and also lower hepcidin mRNA.[40]

GPI-Cp was first characterized in mammalian brain astrocytes.[14] Current experiments detected a relatively low abundance of GPI-Cp in rat cerebellum. Further, unlike spleen and liver, abundance of GPI-Cp in rat cerebellum was not different between CuD and CuA samples. Tissue culture studies with brain glial cells treated with the copper chelator, bathocuproine disulfonate, demonstrated loss of membrane-bound Cp.[38] Thus, potentially a severe copper deficiency in brain could result in decreased expression of GPI-Cp as apparently it does in liver and spleen. Ferroxidase function of brain GPI-Cp may still be compromised by dietary copper deficiency even though the steady-state levels of protein are not impacted. This decreased function may compromise iron efflux from astrocytes to neurons and create a severe functional iron deficiency in brain. Further work on this speculative hypothesis is necessary. Additional work is also necessary to explore the putative function of GPI-Cp in organs such as heart and kidney that express GPI-Cp and currently are not known to have major functions in iron recycling.

Research is needed to determine the mechanism for lower steady-state levels of GPI-Cp protein following copper deficiency, although current data argue against a transcriptional mechanism as steady-state levels of liver and spleen GPI-Cp mRNA were not altered in CuD rats. Seminal work on sCp and copper deficiency previously suggested no transcriptional impact of copper restriction in rat liver.[41,42] Studies on turnover rates of GPI-Cp have not been published. However, enhanced turnover of a similar multicopper oxidase, hephaestin, has been reported under CuD conditions.[43]

As recently reviewed, it is well known that following iron deficiency there is copper accumulation in the liver.[5] Could this accumulation of hepatic copper have an impact on Cp synthesis, secretion and expression? Others found that changing iron status in a rat model had little or no effect on levels and activity of sCP nor expression of mRNA in the liver.[44] Current studies evaluated an acute model of iron deficiency in the growing rat. These animals were anemic and had very low levels of iron in liver and spleen. Interestingly, sCP activity was not statistically impacted; however, the abundance of liver GPI-anchored Cp was actually higher in these rats. The state of iron deficiency was readily evident by the up-regulation of TfR in liver, but not in spleen despite very low NHI content. This suggests the possibility that the TfR in spleen is reflecting a lymphocyte population rather than a macrophage population.

Aceruloplasminemia in humans and deletion of *Cp* in mice both lead to eventual tissue iron overload.[45] However, 10–12-week-old *Cp* $-/-$ mice do not display elevated iron in spleen, whereas at one year of age there is nearly five-fold higher spleen iron.[46] Thus, it is not too surprising that spleen of four-week-old CuD rodents in the current experiments also did not display augmented iron

levels, despite marked reduction in spleen GPI-Cp expression. Plasma sCp levels are lower by 60–90% in these CuD rodents.[19] Also, somewhat confusing for the ferroxidase hypothesis of iron efflux is the observation that CuD and *Cp −/−* mice have normal plasma iron levels.[7,10]

In summary, current work in CuD rats and mice documented decreased levels of GPI-Cp in spleen and liver at several ages, using two different diets, and two strains of rats. In rats, loss of GPI-Cp function could not be demonstrated as lower rather than higher levels of NHI were measured. Reduction in GPI-Cp protein level following copper restriction is likely due to increased turnover rather than impaired biosynthesis, similar to observations by others for multicopper oxidases sCp, hephaestin and zyklopen.[20,43,47] Clearly, further research is needed to determine the impact, if any, of lower GPI-Cp on iron flux and the development of anemia when copper is limiting.

Author contributions: EJM and JRP participated in the design of the studies. EJM conducted most of the technical aspects with help from JRP. Both EJM and JRP evaluated data and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr Z Leah Harris, Vanderbilt University, for the generous gift of *Cp+/+* and *Cp−/−* tissues, Dr Mitchell Knutson for ferroportin antibody and Margaret Broderius for molecular biology technical assistance. This work was supported, in part, by National Institutes of Health grants (R01-HD039708 and R03-HD055423) and the Integrated Biosciences Graduate Program.

REFERENCES

1 Prohaska JR. Copper. In: Bowman BA, Russell RM, eds. *Present Knowledge in Nutrition*. Washington, DC: International Life Sciences Institute, 2006:458–70
2 Hart EB, Steenbock H, Waddell J, Elvehjem CA. Iron in nutrition. VII. Copper as a supplemment to iron for hemoglobin building in the rat. *J Biol Chem* 1928;**77**:797–812
3 Osaki S. Kinetic studies of ferrous ion oxidation with crystalline human ferroxidase (ceruloplasmin). *J Biol Chem* 1966;**241**:5053–9
4 Lee GR, Nacht S, Lukens JN, Cartwright GE. Iron metabolism in copper-deficient swine. *J Clin Invest Res* 1968;**47**:2058–69
5 Collins JF, Prohaska JR, Knutson MD. Metabolic crossroads of iron and copper. *Nutr Rev* 2010;**68**:133–47
6 Fox PL. The copper-iron chronicles: the story of an intimate relationship. *Biometals* 2003;**16**:9–40
7 Pyatskowit JW, Prohaska JR. Copper deficient rats and mice both develop anemia but only rats have lower plasma and brain iron levels. *Comp Biochem Physiol C* 2008;**147**:316–23
8 Holmberg CG, Laurell CB. Investigations in serum copper; nature of serum copper and its relation to the iron-binding protein in human serum. *Acta Chem Scand* 1947;**1**:944–50
9 Harris ZL, Klomp LW, Gitlin JD. Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *Am J Clin Nutr* 1998;**67**:972S–7S
10 Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. *Proc Natl Acad Sci USA* 1999;**96**:10812–7
11 Cherukuri S, Tripoulas NA, Nurko S, Fox PL. Anemia and impaired stress-induced erythropoiesis in aceruloplasminemic mice. *Blood Cells Mol Dis* 2004;**33**:346–55
12 Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP, David S. Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. *J Neurosci* 2002;**22**:6578–86
13 Yamamoto K, Yoshida K, Miyagoe Y, Ishikawa A, Hanaoka K, Nomoto S, Kaneko K, Ikeda S, Takeda S. Quantitative evaluation of expression of iron-metabolism genes in ceruloplasmin-deficient mice. *Biochim Biophys Acta* 2002;**1588**:195–202
14 Patel BN, David S. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *J Biol Chem* 1997;**272**:20185–90
15 Patel BN, Dunn RJ, David S. Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. *J Biol Chem* 2000;**275**:4305–10
16 Fortna RR, Watson HA, Nyquist SE. Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions. *Biol Reprod* 1999;**61**:1042–9
17 Mittal B, Doroudchi MM, Jeong SY, Patel BN, David S. Expression of a membrane-bound form of the ferroxidase ceruloplasmin by leptomeningeal cells. *Glia* 2003;**41**:337–46
18 Hellman NE, Gitlin JD. Ceruloplasmin metabolism and function. *Annu Rev Nutr* 2002;**22**:439–58
19 Broderius M, Mostad E, Wendroth K, Prohaska JR. Levels of plasma ceruloplasmin protein are markedly lower following dietary copper deficiency in rodents. *Comp Biochem Physiol C* 2010;**151**: 473–9
20 Holtzman NA, Gaumnitz BM. Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. *J Biol Chem* 1970;**245**:2354–8
21 Madsen E, Gitlin JD. Copper and iron disorders of the brain. *Annu Rev Neurosci* 2007;**30**:317–37
22 Bastian TW, Prohaska JR, Georgieff MK, Anderson GW. Perinatal iron and copper deficiencies alter neonatal rat circulating and brain thyroid hormone concentrations. *Endocrinology* 2010;**151**: 4055–65
23 Prohaska JR. Changes in Cu,Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper-deficient mice and rats. *J Nutr* 1991;**121**:355–63
24 Pyatskowit JW, Prohaska JR. Multiple mechanisms account for lower plasma iron in young copper deficient rats. *Biometals* 2008;**21**: 343–52
25 Torrance JD, Bothwell TH. A simple technique for measuring storage iron concentrations in formalinised liver samples. *S Afr J Med Sci* 1968;**33**:9–11
26 Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978;**87**:206–10
27 Shen F, Ross JF, Wang X, Ratnam M. Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. *Biochemistry* 1994;**33**:1209–15
28 Wiggins JE, Goyal M, Wharram BL, Wiggins RC. Antioxidant ceruloplasmin is expressed by glomerular parietal epithelial cells and secreted into urine in association with glomerular aging and high-calorie diet. *J Am Soc Nephrol* 2006;**17**:1382–7
29 Stasi K, Nagel D, Yang X, Ren L, Mittag T, Danias J. Ceruloplasmin upregulation in retina of murine and human glaucomatous eyes. *Invest Opthamol Vis Sci* 2007;**48**:727–32
30 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**:RESEARCH0034
31 Jenkitkasemwong S, Broderius M, Nam H, Prohaska JR, Knutson MD. Anemic copper-deficient rats, but not mice, display low hepcidin expression and high ferroportin levels. *J Nutr* 2010;**140**:723–30
32 West EC, Prohaska JR. Cu, Zn-superoxide dismutase is lower and copper chaperone CCS is higher in erythrocytes of copper-deficient rats and mice. *Exp Biol Med* 2004;**229**:756–64
33 Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* 1999;**21**:195–9

34 Banha J, Marques L, Oliveira R, Martins Mde F, Paixao E, Pereira D, Malho R, Penque D, Costa L. Ceruloplasmin expression by human peripheral blood lymphocytes: a new link between immunity and iron metabolism. *Free Rad Biol Med* 2008;**44**:483–92
35 Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab* 2005;**1**:191–200
36 Welch KD, Hall JO, Davis TZ, Aust SD. The effect of copper deficiency on the formation of hemosiderin in sprague-dawley rats. *Biometals* 2007;**20**:829–39
37 Nose Y, Kim BE, Thiele DJ. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. *Cell Metab* 2006;**4**:235–44
38 De Domenico I, Ward DM, di Patti MC, Jeong SY, David S, Musci G, Kaplan J. Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J* 2007;**26**:2823–31
39 Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004;**306**: 2090–3
40 Chen H, Huang G, Su T, Gao H, Attieh ZK, McKie AT, Anderson GJ, Vulpe CD. Decreased hephaestin activity in the intestine of copper-deficient mice causes systemic iron deficiency. *J Nutr* 2006;**136**:1236–41
41 McArdle HJ, Mercer JF, Sargeson AM, Danks DM. Effects of cellular copper content on copper uptake and metallothionein and ceruloplasmin mRNA levels in mouse hepatocytes. *J Nutr* 1990;**120**: 1370–5
42 Gitlin JD, Schroeder JJ, Lee-Ambrose LM, Cousins RJ. Mechanisms of caeruloplasmin biosynthesis in normal and copper-deficient rats. *Biochem J* 1992;**282**:835–9
43 Nittis T, Gitlin JD. Role of copper in the proteosome-mediated degradation of the multicopper oxidase hephaestin. *J Biol Chem* 2004;**279**:25696–702
44 Tran T, Ashraf M, Jones L, Westbrook T, Hazegh-Azam M, Linder MC. Dietary iron status has little effect on expression of ceruloplasmin but alters that of ferritin in rats. *J Nutr* 2002;**132**:351–6
45 Harris ZL. Aceruloplasminemia. *J Neurol Sci* 2003;**207**:108–9
46 Meyer LA, Durley AP, Prohaska JR, Harris ZL. Copper transport and metabolism are normal in aceruloplasminemic mice. *J Biol Chem* 2001;**276**:36857–61
47 Chen H, Attieh ZK, Syed BA, Kuo YM, Stevens V, Fuqua BK, Andersen HS, Naylor CE, Evans RW, Gambling L, Danzeisen R, Bacouri-Haidar M, Usta J, Vulpe CD, McArdle HJ. Identification of zyklopen, a new member of the vertebrate multicopper ferroxidase family, and characterization in rodents and human cells. *J Nutr* 2010;**140**:1728–35

(Received August 24, 2010, Accepted December 9, 2010)