Proteins involved in iron metabolism in beef cattle are affected by copper deficiency in combination with high dietary manganese, but not by copper deficiency alone

S. L. Hansen, N. Trakooljul, H.-C. S. Liu, J. A. Hicks, M. S. Ashwell, and J. W. Spears

ABSTRACT: A 493-d study was conducted to determine the impact of a severe, long-term Cu deficiency on Fe metabolism in beef cattle. Twenty-one Angus calves were born to cows receiving one of the following treatments: 1) adequate Cu (+Cu), 2) Cu deficient (−Cu), and 3) Cu deficient plus high Mn (−Cu+Mn). Copper deficiency was induced through the addition of 2 mg of Mo/kg of DM. After weaning, calves remained on the same treatment as their dam through growing (basal diet analyzed 7 mg of Cu/kg of DM) and finishing (analyzed 4 mg of Cu/kg of DM) phases. Plasma Fe concentrations were positively correlated \( (P < 0.01; r = 0.49) \) with plasma Cu concentrations. Liver Fe concentrations were greater \( (P = 0.05) \) in −Cu vs. +Cu calves and further increased \( (P = 0.07) \) in −Cu+Mn vs. −Cu calves. There was a negative relationship \( (P < 0.01; r = -0.31) \) between liver Cu and Fe concentrations. This relationship is likely explained by less \( (P < 0.01) \) plasma ceruloplasmin activity in −Cu than +Cu calves. As determined by real-time reverse transcription-PCR, relative expression of hepatic hepcidin was significantly downregulated (>1.5 fold) in −Cu compared with +Cu calves \( (P = 0.03) \), and expression of hepatic ferroportin tended \( (P = 0.09) \) to be downregulated in −Cu vs. +Cu. In the duodenum, ferritin tended to be upregulated in −Cu vs. +Cu calves \( (P < 0.06) \). No significant change \( (P > 0.20) \) due to Cu-deficiency was detected at the transcriptional level for either isoform of divalent metal transporter 1 (DMT1 mRNA with or without an iron responsive element; \textit{dmt1}IRE and \textit{dmt1}-non\textit{IRE}) in liver or intestine. Duodenal expression of hephaestin and ferroportin protein was not affected by dietary treatment \( (P > 0.20) \). However, duodenal expression of DMT1 protein was less \( (P = 0.04) \) in −Cu+Mn steers vs. −Cu steers. In summary, Cu deficiency alone did affect hepatic gene expression of hepcidin and ferroportin, but did not affect duodenal expression of proteins important in Fe metabolism. However, the addition of 500 mg of Mn/kg of DM to a diet low in Cu reduced duodenal expression of the Fe import protein DMT1.

Key words: cattle, copper, iron, metal transporter

INTRODUCTION

Copper deficiency is a common problem in the United States (USDA, 2000). Copper-deficient animals may also experience Fe deficiency because 2 Cu-dependent ferroxidases, hephaestin and ceruloplasmin, are essential for mobilization of Fe from tissues (Sharp, 2004). Reduced ferroxidase activity causes a buildup of tissue Fe, reducing circulating concentrations of Fe, resulting in the characteristic anemia of Fe deficiency (Sharp, 2004).

Supplementing Mo to cattle fed diets with reduced Cu is an effective way to induce Cu deficiency, as we have previously demonstrated (Hansen et al., 2008, 2009). We have previously reported that increased dietary Mn also further depresses liver and plasma Cu concentrations in cattle already experiencing severe Cu deficiency (Legleiter et al., 2007; Hansen et al., 2009). It is not uncommon to find Mn concentrations greater than 100 mg/kg of DM in many types of forage (Grace, 1973). In humans, increased dietary Mn has been shown to negatively affect absorption of Fe (Rossander-Hulten et al., 1991). Hartman et al. (1955) reported that hemoglobin formation and serum Fe concentrations were adversely affected in young lambs fed 1,000 or 2,000...
Table 1. Ingredient composition of growing and finishing diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Growing phase</th>
<th>Finishing phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>86.88</td>
<td>—</td>
</tr>
<tr>
<td>Ground corn</td>
<td>—</td>
<td>83.78</td>
</tr>
<tr>
<td>Soybean meal (48%)</td>
<td>9.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Cottonseed hulls</td>
<td>—</td>
<td>5.00</td>
</tr>
<tr>
<td>Urea</td>
<td>1.00</td>
<td>0.80</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>Soybean meal (48%)</td>
<td>9.00</td>
<td>7.00</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Trace mineral premix</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Treatment supplement</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

1-Analyzed 7 mg of Cu, 59 mg of Mn, and 765 mg of Fe per kg of DM.
2-Analyzed 4 mg of Cu, 32 mg of Mn, and 52 mg of Fe per kg of DM.
3-Provided per kilogram of premix: 6,600,000 IU of vitamin A; 1,520,000 IU of vitamin D; and 6,600 IU of vitamin E.
4-Provided per kilogram of diet: 30 mg of Zn as ZnSO₄; 0.5 mg of I as Ca(IO₃)₂(H₂O); 0.2 mg of Se as Na₂SeO₃; and 0.1 mg of Co as CoCO₃.
5-Provided 33 mg of monensin (Elanco Animal Health, Greenfield, IN)/kg of DM.
6-A corn silage supplement provided the following treatments: +Cu (10 mg of Cu/kg of DM, 20 mg of Mn/kg of DM); −Cu (20 mg of Mn/kg of DM, 2 mg of Mo/kg of DM); −Cu+Mn (500 mg of Mn/kg of DM, 2 mg of Mo/kg of DM).

mg of Mn/kg of DM compared with those receiving no supplemental Mn.

Although there is a demonstrable interaction between Cu, Mn, and Fe, essentially no information exists concerning the molecular mechanisms behind this interrelationship in ruminants. Therefore, the objectives of the present study were 2-fold: 1) to determine if proteins known to be essential to Fe metabolism in rodents and humans are present in the bovine, and 2) to determine the effects of a long-term, severe Cu deficiency in the presence or absence of increased dietary Mn on gene and protein expression of these components of Fe metabolism.

MATERIALS AND METHODS

Care, handling, and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee before initiation of the trial.

Animals and Experimental Design

Twenty-one Angus steers (n = 12) and heifers (n = 9) averaging 38.9 ± 2.4 kg at birth were used in this study. Calves were born to cows that had received dietary treatments for at least 410 d by calving, and calves in the present study represent the second set of offspring born to these cows (Legleiter et al., 2008). After the first set of calves were born, cows were moved into a covered barn with slotted floor pens and outdoor runs and remained in these pens until the second set of calves was weaned. During this time cows were fed a corn-silage based diet and received the same Cu, Mn, and Mn treatments that the weaned calves later received.

Dietary treatments included the following: 1) 10 mg of supplemental Cu/kg of DM (Cu adequate, +Cu; n = 6), 2) no supplemental Cu plus 2 mg of Mo/kg of DM (Cu deficient, −Cu; n = 8), and 3) no supplemental Cu plus 2 mg of Mo/kg of DM and 500 mg of Mn/kg of DM (Cu deficient plus Mn, −Cu+Mn; n = 7). Diets were formulated to meet or exceed all NRC recommendations (NRC, 1996) with the exception of Cu. Ingredient and chemical compositions of the basal diets are shown in Table 1. Experimental induction of Cu deficiency in treatments −Cu and −Cu+Mn was achieved through the addition of sodium molybdate (2 mg of Mo/kg of DM) in combination with a reduced Cu diet. Supplemental Mo results in the formation of ruminal thiomolybdates, which bind dietary Cu in the rumen and reduce Cu absorption (Suttle, 1991). Supplemental minerals were provided as Cu from tribasic Cu₂(OH)₃Cl (Micronutrients, Indianapolis, IN), Mn from MnSO₄·H₂O (Sulfamex, Veracruz, Mexico), and Mo from Na₂MoO₄ (Eastern Minerals Inc., Henderson, NC).

Average calf birth date was considered d 0 of the present study, and all days mentioned are based on average calf age on that day. Birth and management of calves through weaning has been described elsewhere (Hansen et al., 2009). Briefly, calves were born over a 36-d period and were weaned at 183 d of age. Calves remained on the same dietary treatment as their dam. After weaning, calves were bunk fed dietary treatments for a period of 34 d. On d 217 calves were moved to pens with electronic Calan gate feeders (American Calan, Northwood, NH) and were fed individually through a 136-d growing phase and a 139-d finishing phase. Calves were fed once daily at 0700 h, with feed amounts based on what they would consume in a 24-h period.

Jugular blood samples were collected at weaning (d 183) and at 2 points each during the growing (d 241 and 297) and finishing (d 422 and 459) phases for analysis of plasma Fe. Blood samples for hematocrit and ceruloplasmin activity were collected on d 421, 490, and 499, respectively. Blood (6 mL) was collected in vacuum tubes containing 10.8 mg of K₂EDTA designed for trace mineral analysis (Becton Dickenson, Rutherford, NJ), transferred on ice to the laboratory, and centrifuged at 1,200 × g for 20 min at 20°C. Plasma was removed and stored at −20°C until analysis. Liver biopsy samples were obtained as described by Engle and Spears (2000) once calves were large enough to adequate sample starting on d 114, at weaning d 183, at a point during the growing phase d 297, and at a point during the finishing phase d 422, for liver Fe determination.

Tissue Collection and Analytical Procedures

On d 493 of the study calves were slaughtered at a commercial abattoir. Calves were stunned via captive
Proteins involved in iron metabolism

and Immunoblotting Procedures

Protein Extraction

DNA Analysis

To validate sample identity from all tissues collected at the slaughterhouse, DNA from tissues was compared with DNA isolated from jugular blood samples previously collected during the trial. Isolation of DNA from 30 mg of tissue was conducted using the Gentra Puregene DNA Purification kit (Qiagen, Valencia, CA). The DNA was PCR amplified with 7 fluorescently labeled microsatellite markers and unique polymorphic markers were selected for animal differentiation. Fragment analysis for 5 markers was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and alleles were sized using Genemapper v3.7 software (Applied Biosystems).

Protein Extraction and Immunoblotting Procedures

Only tissues collected from steers were utilized in the analysis of RNA and protein expression because of unbalanced numbers of steers and heifers. A total of 12 steers were used, with the following breakdown by treatment: n = 3 (+Cu); n = 4 (−Cu); and n = 5 (−Cu+Mn). Isolation of proteins from duodenal scrapings, protein separation, and immunoblotting procedures have been described (Hansen et al., 2009). After blocking, membranes were probed with 1 of the following antibodies overnight at 4°C: monoclonal mouse anti-divalent metal transporter 1 (DMT1; Novus Biologicals, Littleton, CO; catalog number H00004891-M01; 2 µg/mL; 74% homology between antigen sequence and predicted Bos taurus DMT1 sequence), or affinity purified polyclonal rabbit anti-ferroportin (Alpha Diagnostics, San Antonio, TX; catalog number MTP11-A; 1 µg/mL; 84% homology between antigen sequence and predicted Bos taurus ferroportin sequence). For determination of hephaestin concentrations, membranes were probed for 1 h at room temperature using a polyclonal mouse anti-hephaestin (Novus Biologicals, catalog number H00009843-A01; 1:1,500 dilution; 63% homology between antigen sequence and predicted Bos taurus hephaestin sequence). A partial recombinant mouse di-valent metal transporter 1 protein (Novus Biologicals) served as the positive control for DMT1 analysis, and water served as the negative control for all proteins. After incubation with the primary antibody, membranes were washed several times in PBS containing 0.05% Tween 20, incubated for 30 min with the appropriate alkaline phosphatase-linked secondary antibody, and then washed several times with the PBS-Tween 20 wash. Membranes were rinsed 2 times with water and then visualized using Lumi-Phos WB (Pierce, Rockford, IL). Images were captured on autoradiography film (CL-XPosure Film, Pierce) and band densities were semi-quantified using Image Quant TL software (Amersham Biosciences, Piscataway, NJ). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed with β-actin (Abcam, Cambridge, MA; 1:5,000 dilution for 1 h at room temperature) as a loading control. Results are reported as β-actin adjusted relative optical intensities in arbitrary units.

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated from tissue samples (stored at −80°C) using a VDI 25 homogenizer with a S100NK-7 dispersion tool (VWR International, West Chester, PA) and an RNeasy Mini kit following the manufacturer’s protocol with on-column DNase digestion (Qiagen). The RNA concentration was determined using a NanoDrop spectrophotometer (Wilmington, DE). Integrity of RNA was assessed by agarose gel electrophoresis. Real-time PCR using the RNA samples as template for the rpl4 amplification was carried out to confirm that there was no genomic DNA contamination. First-strand cDNA was reverse transcribed from 4 µg of total RNA with a SuperScript III First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed in triplicate on an iQ5 Real Time PCR detection System (Bio-Rad, Hercules, CA). A 20-µL PCR reaction containing 80 ng (total RNA) cDNA, 10 pmol each of forward and reverse primers, and 1X SYBR Green Master Mix (Qiagen) was real-time monitored for 40 cycles (95°C for 10 s and 56°C for 30 s) with an initial denaturation at 95°C for 10 min and a final melting curve analysis. Primers (Table 2) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) except for the rps9 (Janovick-Guretzky et al., 2007). Primer efficiency was evaluated with a cDNA serial dilution. Specific amplification was confirmed by melting curve analysis and gel electrophoresis. A Ct value was transformed to a relative quantity using the 2−deltaCt method (Livak and Schmittgen, 2001) where the greatest quantity was scaled to 1. The relative expression was then normalized against the most stably expressed (housekeeping) gene in each tissue (rps9 and rpl4 genes for intestine and liver, respectively).

Statistical Analysis

Statistical analysis of all data was performed by ANOVA for a completely randomized design using the
Table 2. Real-time RT-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>GenBank GI number</th>
<th>Primer sequence forward (5’-3’)</th>
<th>Primer sequence reverse (5’-3’)</th>
<th>Product length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>dmt1_3-IRE</td>
<td>21583521</td>
<td>CAGTTGCTGCAAGAAAATGTT</td>
<td>CCATGATGAAGTGCAGAAGGT</td>
<td>146</td>
</tr>
<tr>
<td>hepcidin</td>
<td>165372028</td>
<td>CCTCGACAGAAGAAGCTGGA</td>
<td>CCACTCCACACAGCAGTTC</td>
<td>113</td>
</tr>
<tr>
<td>rps9</td>
<td>62460479</td>
<td>CGTTTCTGCATTTGGACTGA</td>
<td>GAGCATCTTGTGCATAGGGA</td>
<td>114</td>
</tr>
</tbody>
</table>

1Primer were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
2Rps9 primers were reported by Janovick-Guretzky et al. (2007).

MIXED procedure (SAS Institute Inc., Cary, NC). Individual animal served as the experimental unit in all analysis. For analysis of duodenal mineral concentrations, the model included the fixed effect of treatment and sex. Differences among treatment means were separated using single degree of freedom orthogonal contrasts. The comparisons made included +Cu vs. −Cu and −Cu vs. −Cu+Mn. Plasma and liver Fe data were analyzed as repeated measures, and the model included the fixed effects of treatment, sex, time, and the appropriate interactions. When a treatment × time interaction was observed (P ≤ 0.15), data were further analyzed by sampling day. Interactions that were not significant (P > 0.15) for the measurement of interest were removed from the model. When treatment was significant, the previously stated contrasts were also used for analysis of these data. Simple Pearson correlation coefficients for the relationship between plasma Cu and Fe, and liver Cu and Fe were computed using the CORR procedure of SAS. Means of normalized relative mRNA abundance and protein expression were separated using single degree of freedom orthogonal contrasts, the model included the fixed effects of treatment, sex, and the appropriate interactions. Because of small numbers of animal, tendencies for all data (P ≤ 0.15) are discussed. Data presented are least squares means ± SEM.

RESULTS

Plasma and Liver Measurements

Plasma and liver Cu concentrations have been previously published (Hansen et al., 2009). Briefly, plasma Cu concentrations across all sampling day averaged 1.07, 0.22, and 0.12 mg/L for +Cu, −Cu, and −Cu+Mn, respectively. Plasma Fe concentrations over the course of the study were greater (P = 0.05) in heifers compared with steers (1.60 vs. 1.46 mg of Fe/L, respectively) and were affected by treatment (P = 0.01), time (P = 0.01), and treatment × time (P = 0.02; Table 3). Plasma Fe concentrations at weaning (d 183) were less (P = 0.01) in −Cu calves compared with +Cu calves and did not differ (P = 0.79) between −Cu and −Cu+Mn calves. Plasma Fe was less during the growing phase (d 241, P = 0.03; d 297, P = 0.01) in −Cu calves compared with +Cu calves, whereas the addition of increased Mn to the reduced Cu diet (−Cu+Mn) tended (P = 0.06) to further reduce plasma Fe concentrations compared with −Cu on d 241 and did reduce plasma Fe compared with −Cu on d 297 (P = 0.05). During the finishing phase (d 422) plasma Fe concentrations increased in calves receiving the −Cu diet and were not different (P = 0.80) from +Cu calves at this time point, whereas plasma Fe in calves receiving the −Cu+Mn diet remained less (P = 0.04) than −Cu calves. Interestingly, no differences (P = 0.19) in plasma Fe concentrations among treatments were observed on d 459 of the study. Plasma Cu and Fe concentrations were positively correlated (r = 0.49; P = 0.001). Hematocrit (means ± SEM) on d 422 did not differ (P = 0.9) between +Cu (32 ± 1.4%) and −Cu (32 ± 1.3%) calves, but was less (P = 0.04) in −Cu+Mn (28 ± 1.3%) compared with −Cu calves. Ceruloplasmin activity (mean absorbance ± SEM) on d 490 was dramatically less (P = 0.001) in −Cu vs. +Cu calves (0.042 ± 0.014 and 0.244 ± 0.016 for −Cu and +Cu, respectively) and did not differ (P = 0.62) between −Cu and −Cu+Mn (0.039 ± 0.016).

Tissue Mineral Concentrations

Liver Fe concentrations were greater (P = 0.05) in −Cu calves vs. +Cu calves and tended to be greater (P = 0.07) in −Cu+Mn calves vs. −Cu calves (Table 4). Liver Fe concentrations were affected by time (P = 0.001) and tended (P = 0.12) to be affected by a time × treatment interaction. When analyzed by sampling day, Fe concentrations in the liver did not differ due to treatment on d 114 and 183, but tended to be less (P = 0.07) in +Cu calves compared with −Cu calves and greater (P = 0.07) in −Cu+Mn calves compared with −Cu calves on d 297. Liver Fe concentrations did not differ (P = 0.44) due to dietary treatment on d 422, which may be related to the much decreased Fe concentrations in the finishing diet (52 mg of Fe/kg of DM) relative to the growing diet (765 mg of Fe/kg of DM). Liver Cu and Fe concentrations were negatively correlated (r = −0.31; P = 0.002). Duodenal Cu concentrations were less (P = 0.01) in −Cu calves compared with
+Cu calves and did not differ \((P = 0.98)\) between the Cu-deficient treatments (Table 5). Mucosal Mn concentrations did not differ between +Cu and −Cu calves, but were greater \((P = 0.01)\) in −Cu+Mn calves compared with −Cu calves. Concentrations of Fe in mucosal scrapings did not differ \((P = 0.68)\) among treatments.

### Liver and Duodenal Gene Expression

Hepatic hepcidin expression was downregulated 1.6 fold \((P = 0.03)\) in −Cu steers compared with +Cu steers (Table 6). Expression of ferroportin in liver of −Cu steers was 72% that of expression in +Cu steers \((P = 0.09)\). No differential \((P > 0.4)\) hepatic expression of dmt1 non-IRE, dmt1 IRE, or ferritin was observed between −Cu and +Cu steers. No differences in hepatic expression of dmt1 non-IRE, dmt1 IRE, ferroportin, and ferritinin between −Cu and −Cu+Mn was observed; however, there was a weak tendency \((P = 0.15)\) for hepcidin expression to be less in −Cu+Mn (67% of −Cu steers). In duodenal scrapings (Table 7), ferritin tended \((P = 0.06)\) to be upregulated 1.6 fold in −Cu steers vs. +Cu steers. Transcriptional abundances of duodenal dmt1 non-IRE, dmt1 IRE, and ferroportin did not differ \((P > 0.6)\) between −Cu and +Cu steers nor between −Cu and −Cu+Mn steers.

### Duodenal Protein Levels

Duodenal levels of hephaestin and ferroportin protein were not different \((P > 0.45)\) among treatments (Figure 1). Bovine intestinal hephaestin migrated to an apparent molecular weight of approximately 150 kDa and intestinal ferroportin to an apparent molecular weight of approximately 60 kDa. Levels of duodenal DMT1 protein were less \((P = 0.04)\) in calves receiving the −Cu+Mn diet compared with −Cu calves and did not differ between +Cu and −Cu calves (Figure 1). The DMT1 had an apparent molecular weight of approximately 55 kDa.

### DISCUSSION

In this study we demonstrate the presence of DMT1, ferroportin, and hephaestin, proteins important in Fe metabolism, in bovine duodenum for the first time. Additionally, this work is, to our knowledge, the first to report on the expression of dmt1 IRE, dmt1 non-IRE, ferroportin, and hepcidin in the bovine. A limitation to the present study is the relatively few animal numbers included in the statistical analysis of gene and protein expression; therefore, the reader is encouraged to use caution when interpreting the effect of dietary Cu and Mn on expression of these genes and proteins in the...
bovine. Because of the established interaction between Fe and Cu metabolism in cattle, future research in this area utilizing more experimental units is warranted.

It is well accepted that the metabolism of Fe and Cu are closely related (Sharp, 2004). Two multi-copper oxidases, ceruloplasmin and hephaestin, are necessary for Fe to be mobilized out of the liver and intestine, respectively (Osaki et al., 1971; Chen et al., 2006). In the present study, −Cu and −Cu+Mn calves exhibited severe Cu deficiency, as evidenced by extremely reduced plasma Cu concentrations, reduced ceruloplasmin activity, and decreased liver Cu concentrations (approximately 6 mg/kg of DM; Hansen et al., 2009). Limited ceruloplasmin activity probably prevented Fe from being mobilized out of the liver, causing Fe to accumulate in the liver and limiting Fe availability for extrahepatic tissues. There was a strong negative correlation between liver Cu and liver Fe concentrations, with decreased liver Cu concentrations leading to increased Fe concentrations. It appears that the greatest impact of Cu deficiency on liver Fe accumulation was during the growing phase when the diet contained a rather high amount of Fe (765 mg/kg of DM). In the present study, −Cu and −Cu+Mn calves exhibited severe Cu deficiency, as evidenced by extremely reduced plasma Cu concentrations, reduced ceruloplasmin activity, and decreased liver Cu concentrations (approximately 6 mg/kg of DM; Hansen et al., 2009). Limited ceruloplasmin activity probably prevented Fe from being mobilized out of the liver, causing Fe to accumulate in the liver and limiting Fe availability for extrahepatic tissues. There was a strong negative correlation between liver Cu and liver Fe concentrations, with decreased liver Cu concentrations leading to increased Fe concentrations. It appears that the greatest impact of Cu deficiency on liver Fe accumulation was during the growing phase when the diet contained a rather high amount of Fe (765 mg/kg of DM), perhaps allowing for a more rapid buildup of hepatic Fe.

Because of reduced export of Fe from the liver, plasma Fe concentrations of −Cu and −Cu+Mn calves were decreased. It has been suggested that plasma Fe concentrations of less than 1.32 mg/L may be indicative of Fe deficiency in beef cattle (Puls, 1994). Based on this index, −Cu+Mn calves were at least marginally Fe-deficient on 4 out of the 5 sampling dates and were only slightly over this threshold on d 422. Calves receiving the −Cu diet averaged approximately 1.47 mg/L of Fe/L of plasma over the course of the study, but had plasma Fe concentrations less than 1.32 mg/L on d 183 and 241. Plasma Fe concentrations were normal for calves receiving the Cu-adequate diet.

Calves receiving the −Cu+Mn diet had decreased plasma Cu concentrations compared with −Cu calves and appeared to suffer from a more extensive Cu deficiency (Hansen et al., 2009). The increased severity of Cu deficiency may have more negatively affected the Fe status of −Cu+Mn calves compared with calves receiving the Cu-deficient diet alone, as evidenced by decreased plasma Fe concentrations and reduced hematocrit on d 422. Increased dietary Mn may have antagonized intestinal Cu absorption or interacted with Cu metabolism in some other manner to further exacerbate the effects of a decreased Cu, Mo-supplemented diet on Cu status of these calves.

Alternately, supplementation with increased dietary Mn may have directly interfered with Fe metabolism. Hartman et al. (1955) found that hemoglobin concentrations in weaned lambs were less in lambs supplemented with 1,000 or 2,000 mg of Mn/kg of DM compared with those fed a diet with no supplemental Mn. Additionally, after discontinuation of the treatments hemoglobin concentrations began to rise in both supplemental Mn groups. The authors speculated that excessive dietary Mn compromised hemoglobin formation, possibly

### Table 6. Effect of dietary Cu and Mn concentration on relative mRNA expression of genes involved in Fe metabolism in liver of steers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>SEM</th>
<th>Contrast</th>
<th>Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Cu</td>
<td>−Cu</td>
<td>−Cu+Mn</td>
<td>+Cu vs. −Cu P-value</td>
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<tr>
<td>dmt1 non-IRE</td>
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<td>1.33</td>
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<td>0.29</td>
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<td>0.14</td>
</tr>
<tr>
<td>ferroportin</td>
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<td>1.09</td>
<td>1.18</td>
<td>0.14</td>
</tr>
<tr>
<td>ferritin</td>
<td>1.23</td>
<td>0.94</td>
<td>0.66</td>
<td>0.29</td>
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<tr>
<td>hepcidin</td>
<td>1.76</td>
<td>1.08</td>
<td>0.72</td>
<td>0.17</td>
</tr>
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</table>

1Means presented as relative gene expression using rpl4 as reference gene.
2+Cu, Cu-adequate (n = 3); −Cu, Cu-deficient (n = 4); −Cu+Mn, Cu-deficient plus increased Mn (n = 5).
3Treatment effect (P > 0.15).
4Treatment effect (P < 0.01).
due to decreased Fe availability for heme synthesis because of competition for absorption between Fe and Mn (Hartman et al., 1955).

Recently, in vitro work has suggested that Mn and Fe can be transported by DMT1, and increased concentrations of either mineral have been demonstrated to negatively affect cellular uptake of the other (Gunshin et al., 1997; Garrick et al., 2006). Additionally, the Belgrade rat, which suffers from a spontaneous mutation of DMT1 that renders the protein inactive, has

Table 7. Effect of dietary Cu and Mn concentration on relative mRNA expression of genes involved in Fe metabolism in duodinal scrapings of steers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>SEM</th>
<th>Contrast</th>
<th>P-value</th>
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<td>0.41</td>
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<tr>
<td></td>
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<td></td>
<td>−Cu+Mn</td>
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<tr>
<td>dmt1 IRE</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>−Cu</td>
<td>0.65</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−Cu+Mn</td>
<td>0.67</td>
<td></td>
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</tr>
<tr>
<td>ferritin</td>
<td>+Cu</td>
<td>0.59</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−Cu</td>
<td>0.94</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>−Cu+Mn</td>
<td>0.91</td>
<td></td>
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</tr>
</tbody>
</table>

1Means presented as relative gene expression using rps9 as reference gene.
2+Cu, Cu-adequate (n = 3); −Cu, Cu-deficient (n = 4); −Cu+Mn, Cu-deficient plus increased Mn (n = 5).
3Treatment effect (P > 0.15).
4Treatment effect (P = 0.10).

Figure 1. Effect of dietary Cu and Mn on expression of Fe metabolism proteins in duodenum of steers. A) Hephaestin (treatment, P = 0.45), ferroportin (treatment, P = 0.55), and divalent metal transporter 1 (DMT1; treatment, P = 0.03; −Cu vs. −Cu+Mn, P = 0.04). +Cu, Cu-adequate (n = 3); −Cu, Cu-deficient (n = 4); and −Cu+Mn, Cu-deficient plus increased Mn (n = 5). B) A representative immunoblot of hephaestin, ferroportin, DMT1, and β-actin.
providing evidence for the role of DMT1 in cellular Mn uptake. Belgrade rats are not only very anemic because of limited absorption of dietary Fe, but they also have decreased tissue Mn concentrations compared with their wild type counterparts, suggesting that DMT1 is important in Mn absorption as well (Chua and Morgan, 1997). Limited in vitro work has also suggested that, in addition to Fe and Mn, DMT1 may be responsible for at least some Cu uptake by cells (Arredondo et al., 2003).

Iron metabolism is a closely regulated process that requires the coordinated efforts of several different proteins. In the intestine, DMT1 is essential for adequate Fe uptake by the enterocyte, whereas another Fe transporter, ferroportin, works in concert with the multicopper oxidase hephaestin to mobilize Fe out of the enterocyte and into the bloodstream (De Domenico et al., 2008). In the present study intestinal expressions of both isoforms of dmt1 and ferroportin were not affected by Cu deficiency alone or in the presence of excessive dietary Mn. However, ferritin expression was increased in Cu-deficient calves, suggesting that Cu deficiency resulted in accumulation of Fe in the duodenal mucosa, which may have been due to the expected low activity of hephaestin. However, only a numerical increase in duodenal Fe was observed in −Cu+Mn calves when compared with Cu-adequate calves.

Hepatic expression of hepcidin was downregulated in Cu-deficient calves compared with Cu-adequate calves. Hepcidin, a small liver-derived peptide produced in response to increasing body Fe stores, has been referred to as the master regulator of Fe metabolism. Active hepcidin binds to the cellular Fe exporter ferroportin, present on the basolateral membrane of the enterocyte, causing it to be internalized and degraded, resulting in a drop in cellular Fe export, and thus plasma Fe concentrations (De Domenico et al., 2008). In the present study the downregulation of hepcidin in −Cu and −Cu+Mn calves is likely explained by the moderate deficiency of Fe that both Cu-deficient treatments were experiencing. Similarly, Chen et al. (2006) reported that hepcidin expression was downregulated in Cu-deficient mice and attributed the downregulation to a secondary Fe deficiency induced by a primary deficiency of Cu. In the present study, increased body Fe stores (liver Fe) and decreased systemic Fe (plasma Fe) in both Cu-deficient groups suggest that hepcidin expression is regulated by systemic signals of Fe status rather than local signals of Fe stores.

Hepatic expression of ferroportin tended to be reduced in Cu-deficient calves compared with Cu-adequate calves. Because ferroportin is essential for mobilization of Fe out of the liver, it was expected that expression of this transporter would be increased due to the reduced Fe status of −Cu calves. However, because of the minimal ceruloplasmin activity of −Cu calves, it is possible that ferroportin expression was downregulated to limit export of ferrous Fe, which could not be oxidized to ferric Fe and therefore be unable to bind to transferrin for safe transport via the blood. De Domenico et al. (2007) reported that ceruloplasmin activity was required for stable expression of plasma membrane-bound ferroportin, and that in the absence of ceruloplasmin activity, ferroportin is quickly removed from the plasma membrane and irreversibly degraded.

It is not entirely clear why duodenal DMT1 protein was less in −Cu+Mn calves. It should be noted that the antibody used in the present study was not specific to either isoform of DMT1 and likely recognized both isoforms. Numerous studies have reported that protein levels of DMT1 non-IRE in rodents are not affected by dietary Fe concentration (Frazer et al., 2003; Johnson et al., 2005). Because relative gene expression of either isoform of dmt1 was not different due to dietary treatment, it appears that decreased protein levels may be a result of degradation of DMT1 protein, reduced translation of DMT1 mRNA, or a combination of the 2. Additionally, reduced DMT1 protein may suggest indirect regulation by increased levels of dietary Mn, though it is unclear whether or not this effect would be observed without a severe deficiency of Cu. Because the Cu status of −Cu+Mn calves was more depressed than −Cu calves it is possible that hephaestin activity was severely limited in this treatment group, causing Fe to build up in the enterocyte. Increased Fe stores may have provided negative feedback to DMT1 protein, resulting in degradation of the active protein or transcript to limit additional Fe uptake into the enterocyte.

In conclusion, induction of a long-term, severe deficiency of Cu (−Cu) in growing beef calves reduced Fe status and stimulated alterations in expression of certain genes involved in Fe metabolism including hepatic hepcidin and ferroportin and intestinal ferritin. However, it appears that a mild deficiency of Fe was insufficient to induce changes in intestinal proteins important in Fe metabolism. However, the addition of increased dietary Mn to a diet decreased in Cu (−Cu+Mn) resulted in a more severe Cu deficiency and appeared to induce a more robust deficiency of Fe. Unexpectedly, a decrease in intestinal DMT1 protein was observed in −Cu+Mn steers. In rodents, the role of DMT1 in absorption of Fe, Mn, and Cu may at least partially explain the interactions often observed between these trace elements. The presence of DMT1 in the bovine duodenum provides an intriguing possibility that this protein may be the common link in the pathways of absorption of Fe, Mn, and Cu in the ruminant. Further research is necessary to characterize the interaction among these trace minerals and determine how these interactions may affect the trace mineral requirements of cattle.

LITERATURE CITED


