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Copper supplementation reverses dietary iron overload-induced pathologies in mice

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Abstract

Dietary iron overload in rodents impairs growth and causes cardiac hypertrophy, serum and tissue copper depletion, depression of serum ceruloplasmin (Cp) activity and anemia. Notably, increasing dietary copper content to ~25-fold above requirements prevents the development of these physiological perturbations. Whether copper supplementation can reverse these high-iron-related abnormalities has, however, not been established. The current investigation was thus undertaken to test the hypothesis that supplemental copper will mitigate negative outcomes associated with dietary iron loading. Weanling mice were thus fed AIN-93G-based diets with high (>100-fold in excess) or adequate (~80 ppm) iron content. To establish the optimal experimental conditions, we first defined the time course of iron loading, and assessed the impact of supplemental copper (provided in drinking water) on the development of high-iron-related pathologies. Copper supplementation (20 mg/L) for the last 3 weeks of a 7-week high-iron feeding period reversed the anemia, normalized serum copper levels and Cp activity, and restored tissue copper concentrations. Growth rates, cardiac copper concentrations and heart size, however, were only partially normalized by copper supplementation. Furthermore, high dietary iron intake reduced intestinal ^{64}Cu absorption (~60%) from a transport solution provided to mice by oral, intragastric gavage. Copper supplementation of iron-loaded mice enhanced intestinal ^{64}Cu transport, thus allowing sufficient assimilation of dietary copper to correct many of the noted high-iron-related physiological perturbations. We therefore conclude that high- iron intake increases the requirement for dietary copper (to overcome the inhibition of intestinal copper absorption).

Keywords: Anemia; Cardiac hypertrophy; Ceruloplasmin; Intestine; Copper absorption; Dietary iron loading

1. Introduction

Iron and copper are essential dietary components for humans, and as such, inadequate intakes of both trace minerals are detrimental to human health. Additionally, given the propensity of iron and copper to potentiate the formation of damaging oxygen free radicals, excesses of both metals can lead to tissue damage and associated pathological outcomes. Iron and copper metabolism is thus tightly regulated by sophisticated homeostatic mechanisms. Furthermore, given their similar physiochemical properties, it is not surprising that notable iron-copper interactions have been documented in humans and other mammals over the past several decades [1,2].

Iron-overload diseases, including hereditary hemochromatosis, and iron-loading anemias such as β-thalassemia, are common in humans [3–5]. Rodent models of these human genetic diseases exist, but researchers can also model iron-loading disorders by feeding rodents diets with high concentrations of iron, creating so called dietary iron overload [6–8]. We previously utilized this approach to assess the influence of variable copper intakes on the iron-overload phenotype. Unexpectedly, we noted that high-iron fed rats and mice developed distinct pathologies which were characteristic of copper deficiency, including growth impairment, tissue copper depletion, cardiac hypertrophy, hepatomegaly and depression of serum ceruloplasmin (Cp) activity [9,10]. Notably, supplementing the high-iron diets with extra copper (~25-fold in excess of requirements) prevented the development of most of these physiological perturbations, demonstrating that high-iron intake disrupted copper homeostasis. Interestingly, it has previously been suggested that high iron can antagonize copper [11,12], which is consistent with these observations.

The current investigation was designed to expand upon our previous studies to determine whether copper supplementation could reverse these high-iron-related abnormalities once established. We hypothesized that increasing copper intake would be effective at

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mitigating these pathophysiological disturbances. To test this postulate, we first established the time course of iron loading, and then tested different supplemental copper concentrations for different periods of time, to establish the correct experimental parameters. Having determined the most appropriate experimental design, notably, we demonstrate that increasing copper intake to ~3.5-fold above requirements was effective at preventing and reversing many of the negative outcomes associated with high-iron intake.

2. Materials and methods

2.1. Animals and experimental design

Three-week-old, C57BL/6 mice (Jackson Laboratories; Bar Harbor, ME) were housed in shoe-box cages under standard laboratory conditions (23±2°C, humidity 60–70%, 12 h light/dark cycles). Mice were provided AIN-93G-based diets (Dyets Inc.; Bethlehem, PA) with high (HFe) (~8800 ppm) or adequate (AdFe) (~80 ppm) iron content with adequate copper content (7–8 ppm). These diets were identical to those used in our previous studies [9,10], which established that high-iron feeding for 4 or more weeks caused notable copper-related pathologies in weanling mice. To assess the time course of tissue iron loading and development of associated high-iron-related pathologies, weanling, male mice, were fed the AdFe or HFe diet for 2, 3 or 4 weeks. It was determined that 4 weeks of high-iron feeding was required for the development of more severe copper depletion, so this time period was selected for further experimentation. To test the efficacy of supplemental copper to correct high-iron consumption-related pathologies, copper (II) sulfate pentahydrate (Sigma; cat. # C-6283) was dissolved in purified water at 78.1 or 781 mg/L, which equates to 20 or 200 mg/L of copper, respectively. 200 mg/L Cu was chosen to mimic our previous work, which utilized high-copper diets [9,10], while the 20 mg/L dose was chosen since it was previously established that this copper concentration could reverse copper depletion-related cardiac hypertrophy in rodents [13]. Initially, weanling, male mice were fed the experimental diets for 4 weeks and supplemental copper (at two concentrations) was provided in the drinking water for the last 2 weeks. This experimental approach was designed to determine which supplemental copper concentration would prevent the development of the pathologies associated with high dietary iron intake. The most appropriate copper concentration (i.e., 20 mg/L) was then selected for further studies designed to determine whether supplemental copper could reverse the high-iron-related pathologies once already established. Moreover, given notable sex differences in iron and copper metabolism, we also sought to determine which sex was most appropriate for further experimentation. Male and female mice were thus fed the experimental diets for 4 weeks, with supplemental copper added to the drinking water (at 20 mg/L) for the last 2 weeks. Males were selected for additional experimentation since they developed more severe copper-depletion-related pathologies. Finally, to investigate the ability of supplemental Cu to reverse the high-iron-related pathologies once established, weanling, male mice, were fed the experimental diets for 7 weeks, with supplemental copper (20 mg/L) added to the drinking water for the last 3 weeks. The different feeding/supplementation regimens were designed to answer different questions regarding the influence of high dietary iron intake on copper homeostasis. Experimental mice were weighed weekly, and average food and water consumption was estimated by weighing the amount of food and water provided daily to each cage. This allowed us to assess any differences in growth rates or energy intakes, as well as estimate the amount of copper consumed by the experimental animals. Mice were sacrificed by CO_2 narcosis followed by thoracotomy. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.2. Hematological parameters, and serum and tissue iron and copper quantification

Blood hemoglobin (Hb) and hematocrit (Hct) levels were determined as described previously [14]. Tissue nonheme iron levels were determined using a standard protocol, as previously described [9,14]. Briefly, 10–50 mg (wet weight) tissues samples were digested in 200–1000 μL acid solution (3 mol/L HCl, 10% trichloroacetic acid) and incubated at 65°C for 20 h and then centrifuged. Ten ul of the supernatant was then mixed with 200 μL of chromogen solution (0.01% bathophenanthroline disulphonate, 0.1% thioglycolic acid, 1.5 M sodium acetate in high-purity water). After 10 min incubation at room temperature, absorbance was measured at 535 nm by spectrophotometry. Serum nonheme iron levels were quantified using a standard colorimetric method [10]. To quantify total iron and copper content, tissue and serum samples were digested with HNO_3 in a water bath (initial temperature 95°C) overnight, and then diluted in purified water to keep the final acid concentration between 2–5%. The diluted samples were then filtered (0.45 μm) and analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) (NexIon 300, Perkin-Elmer Corp.; Norwalk, CT). Iron and copper concentrations in tissues were normalized by weight, and in blood, by volume.

2.3. Serum Cp protein levels and activity

Serum Cp protein expression levels were determined by Western blotting. Briefly, serum protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of serum protein (30 μg) were loaded onto 6% SDS-PAGE gels and immunoblotting was carried out using a standard protocol [15]. The primary anti-Cp antibody, which was used at a 1:1000-fold dilution, was from Sigma (cat. # C0911). We previously established the validity of this reagent [16]. The secondary antibody, which was used at a 1:2000-fold dilution, was horseradish peroxidase (HRP)-conjugated donkey, anti-goat IgG (Santa Cruz Biotechnology; cat. # sc-2020). Ponceau S staining and imaging of the stained blots was used to normalize Cp protein levels (Image-Pro Plus). Serum Cp activity was determined by an amine oxidase (*para*-phenylenediamine [*p*PD]) assay, as described previously [16].

2.4. qRT-PCR

Total RNA was isolated with RNAzol RT reagent (Molecular Research Center Inc.; Cincinnati, OH) and SYBR-Green qRT-PCR was performed as previously described [17,18]. Oligonucleotide primers were designed to span large introns to avoid amplification of genomic DNA. Standard-curve reactions validated each primer pair, and melt curves routinely showed single amplicons. Expression of experimental genes was normalized to expression of *Rps18*. Primer sequences were as follows (5′ to 3′): *Rps18*, forward – TTCCAGCACATTTTGCGAGTA, reverse – CACGCCCTTAATGGCAGTGAT; erythropoietin (*Epo*), forward – ATGAAGACTTGCAGCGTGGA, reverse – AGGCCCAGAG GAATCAGTAG; erythroferrone (*Erfe*), forward- ATGGGGCTGGAGAAC, reverse-TGGCATTGTCCAAGAAGACA.

2.5. Copper absorption experiments

These experiments were done in the dark phase (at night), since mice are nocturnal. Mice were fasted for 3 h prior to a ^{64}Cu transport solution being delivered by oral, intragastric gavage. The transport solution (100 μl) contained 20 μCi ^{64}Cu diluted into PBS buffer containing 0.1 N HCl and 3 μM $CuCl_2$. Immediately after gavage, mice were given *ad libitum* access to the same diet (AdFe or HFe) and water [with or without supplemental copper] that they had been consuming, and were sacrificed 8 h later. Radioactivity was measured using a WIZARD^2 Automatic Gamma Counter (Perkin Elmer; Waltham, MA), and counts were corrected by the half-life of ^{64}Cu. ^{64}Cu absorption was calculated as follows: ([disintegrations per minute (dpm) in the entire carcass plus blood (after the entire GI tract was removed)] divided by [total dpm administered by gavage]) × 100. Radioactivity in blood was expressed as dpm/μl and radioactivity in tissues as dpm/mg wet weight.

2.6. Statistical analysis

The data analysis approach that was utilized was developed upon consultation with a biostatistician. Statistical analyses were performed using the JMP (v 13.2.0) and GraphPad Prism (v 7.0.1) computer programs. Data are presented as box-and-whisker plots, displaying the minimum, the lower [25th percentile], the median [50th percentile], the upper [75th percentile] and the maximum ranked sample. All data were first tested for homogeneity of variances by Bartlett's test. If the data were not equally distributed, then data were $\log_{10}$ transformed prior to running statistical analyses (as indicated in the figure legends). The trends in data were then analyzed using a 2-way ANOVA on ranks test. If this analysis showed significant two-way interactions ($P<.05$), Tukey's multiple comparisons post hoc test was utilized to identify groups which varied significantly for a given parameter.

3. Results

3.1. Growth rates, and estimated food consumption and copper intakes

Male mice consuming the HFe diet grew slower than mice consuming the AdFe diet after 2 weeks, with the most significant difference noted after 4 weeks of dietary treatment (Fig. 1A). Providing supplemental copper for the last 2 weeks of a 4-week feeding period did not normalize growth rates when comparing the AdFe and HFe groups, but differences were less pronounced when 200 mg/L copper in drinking water was given to the mice (Fig. 1B). A similar trend was noted in female mice (data not shown; Iron main effect, $P<.0001$). When supplemental copper was provided at 20 mg/L for the last 3 weeks of a 7-week feeding period, the growth rate increased slightly (Fig. 1C).

Mice on both diets consumed an average of ~3 g of food and ~3 ml of water daily, except for when the water contained 200 mg/L of copper, when water intake was ~1.5 ml/d. Based upon average food intake and water consumption, we were able to calculate daily copper intake. We estimated that animals in both dietary groups that had no copper added to the water consumed 0.024 mg copper/day, those with 20 mg/L copper in the water consumed 0.084 mg/day, and those with 200 mg/L