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Copper metabolism in metallothionein-null mice fed a high-zinc diet

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Humans and animals develop low copper (Cu) status when fed diets containing large amounts of zinc (Zn) for an extended period. Current theory states that Zn-induced metallothionein (MT) in the intestinal mucosa binds Cu and prevents its absorption. We tested this theory by using a mouse model with a disruption in the MT gene that renders it incapable of producing functional MT-I and MT-II (MT-null). If the theory is true, then the MT-null mouse should not develop low Cu status when fed a high Zn diet. For 1 week, groups of 4-week-old MT-null and control mice were fed a diet that contained 35 mg Zn and < 1 mg Cu/kg. Each genotype was then divided into two groups each. One group was fed a diet containing 35 mg Zn and 1.5 mg Cu/kg and the other was fed a diet containing 400 mg Zn and 1.5 mg Cu/kg. After 14 days, plasma was harvested and plasma ceruloplasmin amine oxidase (CPAO) activity, a good indicator of Cu status, was determined. The plasma CPAO activity of control mice fed 400 mg Zn/kg diet was 50% of that in similar mice fed 35 mg Zn/kg. Likewise, plasma CPAO activity in MT-null mice fed 400 mg Zn/kg diet was 40% of that in MT-null mice fed 35 mg Zn/kg. These data suggest that MT induction is not required for the development of low Cu status in mice fed a high Zn diet and that the actual mechanism may involve the modulation or inhibition of a Cu transporter protein by Zn. (J. Nutr. Biochem. 9:598–601, 1998) *Published by Elsevier Science Inc. 1998*

Keywords: MT-null mouse; zinc; copper; ceruloplasmin amine oxidase; metallothionein

Introduction

Copper (Cu) status is depressed in animals[1,2] and humans[3,4] when they are chronically fed diets with high concentrations of zinc (Zn). The primary site for this interaction is thought to be the intestinal mucosa, and the current theory states that high luminal Zn content induces mucosal metallothionein (MT).[4] The high concentration of MT then binds Cu and prevents its absorption into the body.[3–5] This implies that the concentration of mucosal Cu should increase as the concentration of MT increases. The observation that this does not happen suggests that Cu is not sequestered by mucosal MT under these conditions and that MT may not be involved in reducing Cu absorption when induced by Zn.[6] However, this speculation is based on circumstantial evidence and needs further substantiation. To examine the hypothesis that excess Zn impacts Cu status through the inducement of mucosal MT, we used the recently developed mouse model that contains a disruption in the MT gene that renders the mouse incapable of producing functional MT-I and MT-II.[7] If the original theory is true, then low Cu status will not be induced by high dietary Zn in this mouse because it has no intestinal MT. We also observed the time course of the development of Cu deficiency in MT-null mice fed a Cu-deficient diet.

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Received April 14, 1998; accepted June 18, 1998.
Presented in part at the Experimental Biology 1997 Meeting in New Orleans, LA. [Reeves, P.G. (1997). Feeding high-zinc diets lowers the copper status in both metallothionein-null and control mice. *FASEB J.* **11,** A182]

0955-2863/98/$00.00
PII S0955-2863(98)00055-2

Materials and methods

This study was approved by the Animal Use Committee of the USDA, ARS, Grand Forks Human Nutrition Research Center, and was in accordance with the guidelines of the National Institutes of Health on the experimental use of laboratory animals.[8]

Experiment I

Sixteen 4-week-old MT-null (129/Sv-Mt1Mt2 < tm1Bri) male mice and 16 4-week-old control (129/Sv-+ < p > + < Try-c > + Mgf-S1f >/J) male mice were purchased from Jackson Laboratories (Bar Harbor, ME USA). They were housed in shoe-box cages with tissue paper for bedding. Unpublished preliminary experiments with similar mice have shown that both the MT-nulls and controls are very resistant to dietary Cu depletion by feeding a high Zn diet. Therefore, for 1 week, all mice in this experiment were fed a diet similar to AIN-93G[9] containing 35 mg of Zn/kg and no added Cu, to aid in reducing their Cu stores. Then each genotype was divided into two groups and fed one of two diets. One diet contained 35 mg of Zn and 1.5 mg of Cu/kg of diet and the other contained 400 mg of Zn and 1.5 mg of Cu/kg. To prevent spillage, diets were fed in small beakers placed inside larger glass bowls, and the diets were changed daily. Deionized water was provided ad libitum.

After 2 weeks on this regimen, blood was taken from the orbital sinus into hematocrit tubes for the determination of plasma ceruloplasmin amine oxidase (CPAO) activity. Plasma CPAO activity is a sensitive indicator of Cu status in mice[10] and was assayed by using a modified method of Schosinsky et al.[11] and Lehman et al.[12] Briefly, 225 μL of buffer containing 0.1 mM sodium acetate, 45 mM glacial acetic acid, and 0.1 mM diethylenetriamine pentaacetic acid (DTPA), pH 5.5, were pipetted into each of two tubes. Plasma (15 μL) from each mouse was pipetted into each of the two tubes. The reaction was begun in all tubes by adding 60 μL of 7.9 mM o-dianisidine dihydrochloride. After incubation for 10 minutes at 37°C, the reaction was stopped in one tube for each mouse by adding 600 μL of 9 M sulfuric acid. The tubes were removed from the water bath and vortexed. Color intensity in each tube was determined at 540 nm in microcuvettes in a Beckman spectrometer. After 60 minutes at 37°C, the second set of tubes was treated similarly. CPAO activity was expressed as units/liter of plasma (U/L), where a unit is equal to μmol/min. This activity value was calculated by subtracting the readings at 10 minutes from those at 60 minutes and dividing by the molar absorption coefficient of 9.6 and the incubation time in minutes, and then multiplying by a factor that expresses the activity on the basis of a liter of plasma.

Experiment II

Twenty-five 4-week-old MT-null male mice and 25 control male mice were purchased from Jackson Laboratories. They were housed in shoe-box cages with tissue paper for bedding. When the mice arrived at our laboratory, they were fed Purina Mouse Diet 5015 (PMI Feeds, Inc., Richmond, IN USA) for 3 days. Five mice from both the MT-null and control groups were killed; plasma was isolated for the measurement of CPAO activity, and the intestinal mucosa isolated for MT determination by the method of Eaton and Cherian[13] that was modified to include 2-mercaptoethanol in the buffer.[14] Fifteen of the remaining mice were fed a diet similar to AIN-93G[9] containing 35 mg of Zn/kg and no added Cu. Over the course of 42 days, 5 mice from each genotype were killed at days 10, 14, and 42, and plasma isolated for measurement of CPAO activity and the intestinal mucosa isolated for MT determination. Five of the remaining mice from each genotype were fed a similar diet containing 6 mg Cu/kg for 42 days.

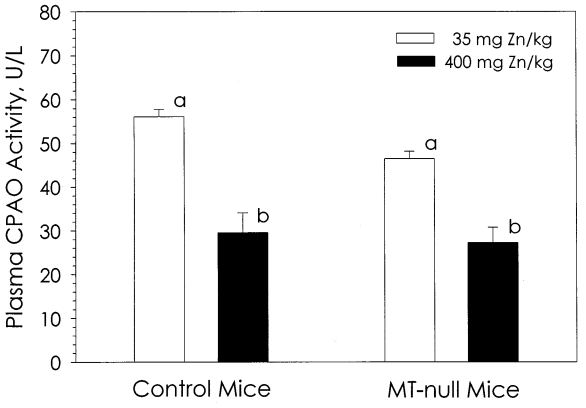

Figure 1 Ceruloplasmin amine oxidase (CPAO) activity in metallothionein (MT)-null and control mice fed diets high in zinc (Zn) concentration (400 mg/kg for 2 weeks). Values are means ± SEM of eight replicates each. Different superscripts between bars within mouse types indicate significant differences ($P < 0.001$) (experiment I).

Statistical analysis

In experiment I, significant differences between means were determined by a two-way analysis of variance (ANOVA) with mouse strain and dietary Zn concentration as the independent variables. The same method was used in experiment II, except the independent variables were time and dietary Zn concentration. The ANOVA and post-hoc test (REGWF) were done by using the Crunch Statistical Package (Crunch Software Corp., Oakland, CA USA). All data are presented as means ± SEM.

Results and discussion

The results of experiment I are shown in *Figure 1*. As expected, plasma CPAO activity in control mice fed 400 mg Zn/kg of diet was only approximately 50% ($P < 0.001$) as high as the values for mice fed 35 mg Zn/kg of diet. Similarly, plasma CPAO activity in MT-null mice fed 400 mg Zn/kg also was reduced and was only approximately 40% ($P < 0.005$) as high as the values for mice fed 35 mg Zn/kg of diet. This smaller difference was because MT-null mice in the low Zn group had somewhat lower CPAO activity than the control group; however, these values were not statistically different ($P > 0.05$).

The concentration of MT was not determined in the intestinal mucosa of the mice in this study. However, in a similar 14-day study conducted previously, high dietary Zn induced mucosal MT by 2.5-fold in control mice, but there was no induction in the MT-null mice (data not shown). Apparently, MT induction is not necessary for the initiation of low Cu status in mice fed high Zn diets.

While working with the MT-null mouse and its control from Jackson Laboratories, we have observed that both types are very resistant to Cu depletion when fed a high Zn diet. We also have observed that this particular MT-null mouse seems to be slightly more resistant to Cu depletion than its control when each is fed a Cu-deficient diet. *Figure 2A* shows the results of experiment II in which the time course of plasma CPAO activity during Cu depletion by diet was followed. Before the feeding trial began, CPAO activity was similar between the two genotypes. However, after only

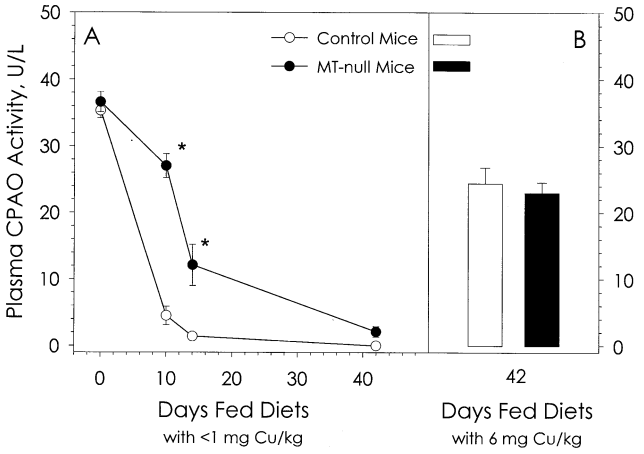

Figure 2 Reduction of copper (Cu) status with time in metallothionein (MT)-null and control mice fed diets low in Cu by using plasma ceruloplasmin amine oxidase (CPAO) activity as a status indicator. Values represent the mean ± SEM of five replicates per point. Panel A represents data for mice fed a low Cu (<1 mg/kg) diet for 42 days with samples taken at intervals. The asterisks indicate that the means between groups at these points in time are significantly ($P < 0.001$) different. Panel B represents data for mice fed a normal Cu (6 mg/kg) diet with plasma samples taken at day 42 only (experiment II).

10 days of feeding the Cu-deficient diet, plasma CPAO activity of the control group was reduced to only 12% of the initial value (chow fed group), and CPAO activity of the MT-null group remained much higher at 74% of the initial value. After 14 days of consuming the Cu-deficient diet, plasma CPAO activity in the control group was reduced to only 4% of the initial value, and activity in the MT-null group was reduced to 32% of the initial value. After 42 days of consuming the Cu-deficient diet, CPAO activity in the control group was near zero, whereas that in the MT-null group was approximately 6% of the initial value, but the difference was not significant. Mean CPAO values between control and MT-null groups were significantly ($P < 0.001$) different at days 10 and 14 but not at days 0 and 42. *Figure 2B* shows plasma CPAO activity in MT-null and control mice fed diets with 6 mg Cu/kg for 42 days. There was no significant difference between groups. However, it was observed that CPAO activity in Cu-fed mice was less at 42 days (11 weeks of age) than at the beginning of the experiment (4 weeks of age). This could have been caused by an artifact of the analysis or by a decrease in CPAO activity in this strain of mouse as it matures.

When using the ^{109}Cd displacement method of Eaton and Cherian[13] with 2-mercaptoethanol, a substantial amount of ^{109}Cd is bound to components in the tissue preparation of MT-null mice. Because this mouse cannot synthesize functional MT-I and MT-II, this may represent binding to ligands that are not related to MT. In our hands, intestinal MT values between MT-null and control mice not fed high Zn diets do not differ. For example, in experiment II, those values calculated as representing intestinal mucosa "MT" over the entire period ranged from 4.8 ± 0.8 to 8.4 ± 1.5 μmol/kg in MT-null mice and 5.5 ± 0.4 to 8.4 ± 0.5 μmol/kg in control mice. There was no significant difference between genotypes.

Although there is a clear delay in the development of Cu deficiency in the MT-null mouse in this study, there may be another side to the story. The speed at which Cu deficiency develops depends on the age of the animals and their previous Cu stores. One of the problems we have had in obtaining large groups of MT-null and control mice from Jackson Laboratories is getting a precise age match between the two genotypes and among individual mice. Having a difference in age of 1 week in the growing animals might affect the initial level of their Cu stores. This in turn might affect how rapidly the animal develops Cu deficiency. Consequently, if the average age of the MT-null mice in this experiment was greater than that of the controls, this could account for the extended period needed to produce Cu deficiency. However, it is not ruled out that the apparent physiologic differences between the two genotypes could have caused this phenomenon as well.

If Zn-induced intestinal MT is not required for lowering Cu status of mice fed high-Zn diets, then what is the mechanism? Previously, we showed that Cu transport was significantly lower in Caco-2 cells incubated for 7 days in a medium containing 50 μmol Zn/L than in cells incubated in a medium containing 7 μmol Zn/L.[15] More recently, we showed a similar effect of only 25 μmol Zn/L medium.[16] In many cell types, including the intestinal mucosa, Cu transport out of the cell is probably regulated by a Cu-transporting P-type adenosinetriphosphatase (ATPase), known as ATP7A or the Menkes protein, with an N-terminal metal-binding amino acid motif.[17,18] Part of the mechanism of action seems to require that the transporter protein be translocated from the Golgi apparatus to the cell membrane,[19] where Cu is then bound to the metal-bind region to initiate transport out of the cell. I suggest that high Zn either alters the translocation process of the transporter component or causes Zn substitution in the metal-binding region of the transporter, thus reducing the efficiency of Cu transport. These two scenarios might imply that Cu would accumulate in the mucosal cell if transport out of the cell is inhibited. However, we have not found this to be the case.[6,16] To complicate matters further, we have also shown that by using the Caco-2 cell as a model, elevated Zn concentrations in the medium will actually enhance the rate of Cu uptake into the cell from the apical side of the cell, but not from the basolateral side;[16] however, Cu does not accumulate in the cell. Whether this phenomenon also occurs in normal enterocytes of animals is not known.

In summary, this study demonstrates that MT is not absolutely necessary for the induction of low Cu status in mice fed a high Zn diet and suggests that the true mechanism may lie in the modulation or inhibition of a Cu transporter by high Zn concentrations. In addition, the data suggest that this particular MT-null mouse is slightly more resistant to Cu depletion induced by feeding a low Cu diet than the control mouse with a similar genetic background, but with a full complement of MT.

Acknowledgments

The author greatly appreciates the technical assistance of Brenda Skinner in performing the chemical analyses, Jim

Lindlauf for mixing the diets, and Denice Schafer and her staff for care of the animals.

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