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Copper deficiency increases metallothionein-I mRNA content selectively in rat liver

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A variety of inducers of metallothionein (MT) synthesis have been identified. Copper loading, in particular, has been shown to increase MT expression in the liver and other organs of rodents. In the present study, the effect of severe dietary copper deficiency on MT expression was determined. Weanling rats were fed a purified diet deficient in copper (0.4 μg/g of diet) or containing adequate copper (5.7 μg/g of diet) for 4 weeks. Mineral analysis revealed that copper concentrations were severely depressed in the liver, heart, and kidney of the animals fed the copper-deficient diet. The mRNA content for MT-I was markedly increased in the copper-deficient liver but not in the heart or kidney. The metallothionein protein concentration, however, was not elevated in either liver or heart and was decreased in the kidney of copper-deficient rats. The results demonstrate that copper deficiency enhances MT-I transcription selectively in the liver of rats. It also suggests that copper is not essential for MT transcription in rats. (J. Nutr. Biochem. 6:572–576, 1995.)

Keywords: copper deficiency; metallothionein; mRNA; liver; heart; kidney

Introduction

Metallothionein (MT) is a low molecular weight metal-binding protein containing a high proportion of cysteine residues but no disulfide bond. MT has been demonstrated to function in protection against metal toxicity, in trace metal homeostasis, and in scavenging of free radicals.[1] The most remarkable biological feature of MT is its inducibility by a variety of agents and conditions including metals, adrenocortical steroids, cytotoxic xenobiotics, cytokines, and stress-producing conditions.[2] The gene regulation of MT is complex. A wide range of transcription factors, including the metal regulatory element, glucocorticoid-responsive element, or interferon-related element,[3] can interact with the MT promoter regions.

Copper loading induces MT synthesis in the liver of rats.[4,5] Copper MT from the liver of copper-injected rats has been isolated and analyzed. The copper-induced MT synthesis is characterized by increased incorporation of [^{35}S]cysteine into the protein[6] and enhanced production of the MT mRNA,[7,8] suggesting that copper induction of MT occurs at the level of gene transcription. The mechanism by which copper induces MT synthesis, however, remains unsolved. It is also unknown whether copper is essential for MT production under a diversity of physiological and pathological conditions. The purpose of this study was thus to examine the effect of dietary copper deficiency on MT induction in rats.

Methods and materials

Diets and animals

A copper-adequate diet was formulated according to Reeves et al. (AIN-93G diet),[9] except that tert-butylhydroquinone was not added. A copper-deficient diet was similarly formulated except for

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the replacement of copper by the corresponding weight of corn starch. Diet analysis for copper yielded values of 5.7 mg of Cu/kg of diet for the copper-adequate diet and 0.4 mg of Cu/kg of diet for the copper-deficient diet. Male weanling Sprague–Dawley rats (46–57 g; Sasco, Lincoln, NE USA) were housed in quarters maintained at 22 to 24°C with a 12-hr light/dark cycle. They were divided into two weight-matched groups having average weights of 52 g each. One group was given free access to the copper-adequate diet and the other to the copper-deficient diet. Rats also had free access to deionized water. These experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*[10]

Tissue sample preparation

After 4 weeks on their respective diets and an overnight fast, each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg of body wt, Vet Labs, Lenexa, KS USA). Blood was withdrawn from the inferior vena cava for erythrocyte counting and plasma assays. The liver and heart were removed, flushed with cold 0.9% NaCl via their major vessels, and divided for subsequent assays. Tissue samples including the liver, heart, and kidney were stored at −20°C for mineral assays and those for MT and mRNA assays were placed in liquid nitrogen then stored at −80°C.

Blood analysis

Hematocrit and hemoglobin content was determined on a Coulter Counter (Model S plus 4, Hialeah, FL USA). A Cobas Fara automated analyzer (Roche Diagnostic Systems, Nutley, NJ USA) was used to determine serum ceruloplasmin.[11]

Analysis of minerals

Trace element contents of tissues were determined by inductively coupled argon plasma emission spectroscopy (Jarrell-Ash, Model 1140, Waltham, MA USA) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide.[12] Assay of dietary copper content was performed by a dry ashing procedure,[13] dissolution in aqua regia, and measurement by atomic absorption spectrophotometry (Perkin Elmer, Model 503, Norwalk, CT USA). Mineral contents of the National Institute of Standards and Technology (NIST) reference samples (#1577a, bovine liver for organs; #1572, citrus leaves for diets) were within the specified ranges by NIST, thus validating the assay procedure.

Analysis of MT mRNA and protein

Total tissue RNA was isolated from quick-frozen tissues by using the RNAzol B method (Cinna/Biotecx, Friendswood, TX USA) and quantified spectrophotometrically. Total RNA, 15 μg, was then subjected to a 1% denaturing agarose gel and transferred to a GeneScreen Plus membrane (Dupont). Hybridization and the wash procedure were conducted by the method described by Church and Gilbert.[14] The probe corresponding to a 1,185 base pair *Hind*III and *Bgl*II fragment of mouse MT-I cDNA was obtained from Dr. R.D. Palmiter at the University of Washington, Seattle, WA.[15] The probe was labeled with [^{32}P]dCTP by using the random-prime method of Feinberg and Vogelstein.[16] After autoradiography, the membrane was stripped and rehybridized with human β-actin cDNA to ensure integrity of the RNA sample and to confirm that equal amounts of RNA had been loaded onto all lanes. Autoradiographic images were scanned and analyzed by using the MCID system from Imaging Research Inc. (Ontario, Canada). Densitometric values were then determined from digitized images of autoradiograms. Values were corrected for background, and expressed as a percent of the average signal in controls. MT concentrations were measured by the Cd/hemoglobin radiometric assay as previously described.[17]

Statistical analysis

Data were analyzed by Student's *t*-test. All experiments were repeated three times and the data were presented from one representative experiment, as the mean ± SE values. Differences between treatments were considered significant at $P < 0.01$.

Results

Characteristics of rats fed the copper-deficient diet were compared with those of rats fed the copper-adequate diet. As shown in *Table 1*, copper concentrations were significantly ($P < 0.01$) depressed in the liver, heart, and kidney of copper-deficient rats. Zinc concentrations were significantly ($P < 0.01$) decreased in the heart and kidney, but not significantly ($P > 0.05$) reduced in the liver. Iron concentrations were also significantly ($P < 0.01$) depressed in the heart and kidney but significantly ($P < 0.01$) elevated in the liver. Other characteristic changes including reduced activity of copper-dependent ceruloplasmin in the plasma, depressed copper- and zinc-dependent superoxide dismutase in the tissues, and decreased hematocrit and hemoglobin concentrations in the blood were found in the rats fed the copper-deficient diet (data not shown). All of these changes under the same experimental conditions have been previously reported[18] and are typically indicative of severe copper deficiency.[19]

The MT mRNA content in the liver, heart, and kidney

Table 1 Status of copper, zinc, and iron in copper-deficient and -adequate rats

Minerals	Liver		Heart		Kidney	
	CuD	CuA	CuD	CuA	CuD	CuA
Cu (nmol/g of dry wt)	14 ± 2*	179 ± 5	69 ± 2*	315 ± 3	167 ± 3*	428 ± 19
Zn (μmol/g of dry wt)	1.00 ± 0.04	1.09 ± 0.02	0.97 ± 0.01*	1.12 ± 0.03	1.33 ± 0.01*	1.40 ± 0.01
Fe (μmol/g of dry wt)	6.63 ± 0.45*	2.98 ± 0.14	4.23 ± 0.07*	5.21 ± 0.10	3.86 ± 0.10*	4.98 ± 0.13

Values are means ± SEs, $n = 9$.
*Indicates significant difference from CuA ($P < 0.01$).

was measured. Total RNA was isolated from each organ and subjected to Northern analysis that utilized [^{32}P]-labeled probe corresponding to a 1,185 base pair *Hind*III and *Bgl*II fragment within the coding region of the mouse MT-I cDNA. As shown in *Figure 1*, the MT-I mRNA concentrations were elevated in the copper-deficient livers ($n = 15$; only two representatives were shown in the figure) by 75.4 ± 3.7 folds ($P < 0.01$), but were not changed in the copper-deficient hearts ($n = 12$) or kidneys ($n = 12$).

To determine whether the elevated mRNA level in the liver correlates with MT protein production, the MT protein content in organs was measured by using the Cd/hemoglobin radiometric assay.[17] The results summarized in *Table 2* show that MT protein concentrations in the copper-deficient liver and heart did not change, but significantly ($P < 0.01$) decreased in the copper-deficient kidney.

Table 2 MT concentrations in liver, heart, and kidney of copper-deficient and -adequate rats

Diet	Liver	Heart	Kidney
CuD ($n = 12$)	46.6 ± 5.3*	4.9 ± 0.4	52.9 ± 1.2†
CuA ($n = 12$)	44.5 ± 5.2	3.9 ± 0.3	94.2 ± 2.6

*Expressed as μg of MT/g of tissue (mean ± SE).
†Significantly different from copper-adequate rats ($P < 0.01$).

Discussion

Induction of MT synthesis by a large number of factors indicates a multifunctional feature of this unique, thiol-rich protein. It has been hypothesized and continually discussed that MT serves as a nonspecific protective factor against metal toxicity, regulates trace metal homeostasis, and participates in antioxidant defenses by scavenging free radicals.[1] Among the MT inducers, copper has been extensively studied, leading to the following hypothesis. As copper is transported to the liver after absorption from the intestine, it binds to thionein. If no binding sites are available on MT, the copper ions promote the production of MT mRNA and protein by interacting with the *trans*-acting factors that bind to the MT genes. The newly synthesized MT then chelates the excess copper ions to remove them from the system. The copper MT may be degraded to release copper for copper-dependent processes and excretion.[20] This coordination between copper and MT synthesis plays an important role in copper homeostasis and biological function. It is also known that under physiological conditions, copper is important in the regulation of MT production.[20]

Many other factors induce MT synthesis.[2] It is unknown whether copper is essential for MT synthesis stimulated by these diverse inducers. Studies by Mercer et al. have demonstrated the relationship between concentrations of copper, zinc, and MT-I mRNA in the liver, and brain of brindled mutant mice,[21] The MT-I mRNA concentration was significantly elevated in the liver of mutant mice between days 12 and 16 after birth, while the copper concentration in the liver was severely depressed. In contrast, copper concentrations in the kidney were elevated, but the MT-I mRNA concentration was not altered. Zinc concentrations were not changed in either liver or kidney of the mutant mice. In addition, the possibility that the brindled mutation causes constitutive activation of MT genes has been eliminated.[21,22] Therefore, some factors other than mutation, copper, or zinc may be involved in the regulation of MT gene expression in brindled mutant mice. In the present study, we developed a severely copper-deprived rat model by feeding the normal animals a copper-deficient diet. Analysis of minerals revealed that copper concentrations in the liver, heart, and kidney were all severely decreased. The MT-I mRNA concentration in the copper-deficient liver was, however, markedly elevated. When compared with the effect on MT-I transcription in the heart and kidney, the copper deficiency-elevated MT-I mRNA in the liver is clearly tissue-specific.

Zinc is an inducer of MT synthesis. In the present study, we found that zinc concentrations were significantly depressed in the heart and kidney, but were not significantly altered in the liver of copper-deficient rats, indicating that the enhanced synthesis of MT-I mRNA in the liver did not result from changes in the status of zinc. In contrast, the iron concentration was markedly increased in the liver but significantly decreased in the heart and kidney of the copper-deficient rats. It has been known that iron can bind to MT in vitro, but does not do so in vivo, and changes in iron status do not have a major effect on MT production.[23] At very high dietary iron intakes, increased synthesis of zinc MT has been observed,[24] which has been ascribed to a possible stress response.

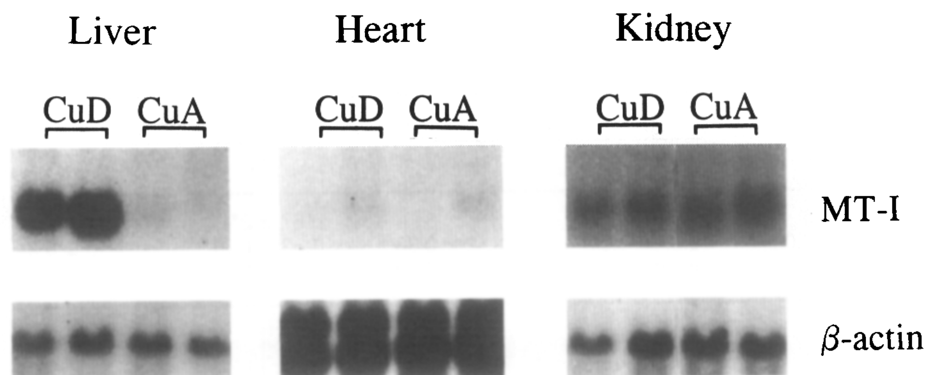

Figure 1 Representative autoradiography of the Northern blot and analysis of MT-I mRNA in the liver, heart, and kidney of copper-deficient (CuD) and -adequate (CuA) rats. The experimental procedure is described in the text. Autoradiographic image analysis of all the blots from three repeated experiments indicates that the amount of mRNA in the CuD livers is 75 fold higher than in the CuA livers ($n = 15$). Such as change was not detected in the hearts ($n = 12$) or kidneys ($n = 12$).

It has been suggested that the enhanced synthesis of MT-I mRNA in the liver of brindled mutant mice resulted from stress.[21] Copper deficiency induces many biochemical changes and pathophysiological consequences in many organs including liver, heart, and kidney. Several studies suggest that oxidative stress is involved in the copper deficiency-induced pathological processes. Enhanced lipid peroxidation in copper-deficient tissues and inhibition of copper deficiency-induced defects by antioxidants were observed.[25–27] Because MT is an important antioxidant, participating in cellular protection against oxygen-free radical-induced damage, up-regulation of MT synthesis may reflect a general adaption to the copper deficiency-induced oxidative stress. However, our previous studies[18] have shown that under the same experimental conditions, a higher degree of oxidative damage occurs in the heart than in the liver of copper-deficient rats. Therefore, the extent of oxidative stress alone cannot account for the elevated MT-I mRNA concentration in the liver. Mechanisms responsible for the selective response of liver MT gene transcription to copper deficiency need to be further investigated.

Another observation made in the present study is that a corresponding increase in MT protein in the copper-deficient liver was not detected. MT is transported from the liver into the bile and blood.[28] Accumulation of MT in the kidney would thus be expected if such a transport occurs. However, MT transport does not account for the undetected elevation of MT content corresponding to the increased MT mRNA, because the MT concentration in the kidney did not increase but significantly decreased. Early studies on brindled mutant mice have shown that hepatic MT synthesis was reduced in the brindled neonate,[29] which has been attributed to the low hepatic copper concentration in the mutant.[30] This led to a conclusion that copper is the most likely regulator of hepatic MT synthesis in the neonatal mouse liver.[29,30] These early studies, however, did not examine the concentration of MT mRNA. In contrast to the early conclusion, studies by Mercer et al. suggest that hepatic copper is not regulating MT mRNA production.[21] In the present study, we measured both MT mRNA and protein concentrations. The results, together with previous studies,[21,29,30] suggest that copper is not essential for MT mRNA production, but it may be required for MT translation.

In summary, this study examined the effect of a severely depressed copper concentration on MT induction in rats. The results demonstrate that copper deficiency induces MT gene transcription selectively in the liver, which is not accompanied by elevation of MT protein concentrations. This selective induction of MT-I mRNA in the liver unlikely results from changes in the status of copper and zinc in the tissue. Furthermore, the data suggest that although copper is not essential for MT transcription, it may be required for the translation.

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