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RESEARCH ARTICLES

Changes in copper and zinc status and response to dietary copper deficiency in metallothionein-overexpressing transgenic mouse heart☆

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Abstract

Previous studies have shown that cardiac-specific overexpression of metallothionein (MT) inhibits progression of dietary copper restriction-induced cardiac hypertrophy. Because copper and zinc are critically involved in myocardial response to dietary copper restriction, the present study was undertaken to understand the effect of MT on the status of copper and zinc in the heart and the subsequent response to dietary copper restriction. Dams of cardiac-specific MT-transgenic (MT-TG) mouse pups and wild-type (WT) littermates were fed copper-adequate (CuA) or copper-deficient (CuD) diet starting on the fourth day post delivery, and the weanling mice were continued on the same diet until they were sacrificed. Zinc and copper concentrations were significantly elevated in MT-TG mouse heart, but the extent of zinc elevation was much more than that of copper. Dietary copper restriction significantly decreased copper concentrations to the same extent in both MT-TG and WT mouse hearts, and decreased zinc concentrations along with a decrease in MT concentrations in the MT-TG mouse heart. Copper deficiency-induced heart hypertrophy was significantly inhibited, but copper deficiency-induced suppression of serum ceruloplasmin or hepatic Cu,Zn-SOD activities was not inhibited in the MT-TG mice. These results suggest that elevation in zinc but not in copper in the heart may be involved in the MT inhibition of copper deficiency-induced cardiac hypertrophy.

Keywords: Copper; Zinc; Heart hypertrophy; Metallothionein; Transgenic mice

1. Introduction

Previous studies using mouse model have demonstrated that dietary copper restriction causes heart hypertrophy with defected function [1–3]. Cardiac-specific overexpression of metallothionein (MT) in transgenic mice inhibits progression of heart hypertrophy induced by dietary copper deficiency [4]. MT is a metal binding protein, and under physiological conditions, MT predominantly binds to zinc [5,6]. However, zinc can be replaced by copper or other metals such as cadmium under the condition of overload of these metals [7]. Because the status of copper and zinc in the heart greatly affects myocardial response to dietary copper restriction, the inhibitory effect on copper deficiency-induced heart hypertrophy in the cardiac-specific MT-overexpressing transgenic mice may relate to MT manipulation of the status of these minerals. However, it is unknown what are the metals that are bound to MT in the transgenic mouse heart in that the expression of MT is controlled by cardiac α-myosin heavy chain promoter [8].

The present study was undertaken to specifically examine the effect of MT overexpression on copper and zinc status in the heart and the subsequent response to dietary copper restriction. In particular, we focused on possible correlation between MT manipulation of copper status and the inhibition of copper deficiency-induced heart hypertrophy in the cardiac-specific MT-overexpression transgenic mice. The results obtained demonstrate that although copper concentrations were elevated in the MT-overexpressing transgenic mouse heart, copper deficiency caused depletion of copper in the heart to the same level between the transgenic mice and the wild-type (WT) controls. However, the elevation of zinc in the

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MT-overexpressing transgenic mouse heart was more predominant. These findings thus suggest MT manipulation of zinc rather than copper status in the heart is more likely involved in the inhibition of copper deficiency-induced heart hypertrophy.

2. Materials and methods

2.1. Animals and treatment

FVB mice were originally obtained from Harlan Bioproducts for Science, Inc., (Indianapolis, IN, USA) and maintained at the University of Louisville animal facilities. The cardiac-specific MT-TG mice were produced from the FVB stain as described previously [8]. The MT-TG mice were then bred with the same strain of WT mice. They were housed in plastic cages at 22°C on a 12-h light/dark cycle. Dams of the pups (both heterozygous MT-TG mice and their WT littermates) were fed copper-deficient (CuD) or copper-adequate (CuA) diet starting on the fourth day post delivery. The pups were weaned on the 21st day after birth and the weanling mice were continued on the same diet as their dams until they were sacrificed at 3, 4 or 5 weeks after CuD feeding (combined pre- and post-weanling feeding). The number of mice used at each time point for each treatment group was 6. The animals had free access to doubly distilled water. The CuA and CuD diets (AIN-93 diet) were prepared according to Reeves et al. [9] and the primary ingredients were cornstarch (53%), casein (20%), sucrose (10%) and soybean oil (7%). Vitamins and minerals were provided in the diet exactly as described previously [9]. The CuA diet included an addition of 6 mg of Cu/kg diet in the form of $CuSO_4$, and the corresponding weight of cornstarch was added to the CuD diet. Analyses of the diets for Cu concentrations yielded 6.089 mg Cu/kg diet for CuA and 0.348 mg Cu/kg diet for CuD diet. All procedures were approved by the AAALAC-certified University of Louisville Institutional Animal Care and Use Committee.

2.2. Tissue harvest

At the end of the feeding experiment and after an overnight fast, each animal was anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight, Vet Labs, Lenexa, KS, USA). Blood was withdrawn from the abdominal vena cava and serum was separated with a Serum Separator (Becton Dickenson, Inc., Rutherford, NJ, USA) within 30 min. An incision was made in the inferior vena cava and the heart was perfused with cold 0.9% NaCl. The heart was then removed, opened, washed, dried with paper tissue and weighed. The left ventricle was used for copper, zinc and MT determinations. The liver was also perfused with cold 0.9% NaCl through the portal vein, and portions of the liver were excised. All the tissue samples were either used immediately or placed in liquid nitrogen, then stored at −80°C for later analysis.

2.3. Mineral concentrations

Mineral concentrations in the heart were measured using inductively coupled argon plasma emission spectroscopy (model 35608, Thermo ARL-VG Elemental, Franklin, MA, USA) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide [10]. Dietary Cu concentrations were analyzed by using a dry-ashing procedure, which was followed by dissolution of the residue in aqua regia and measurement by atomic absorption spectrophotometry (model 503, Perkin Elmer, Norwalk, CT, USA). Trace element contents of National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) reference samples were within the specified ranges established by NIST, thus validating our assay procedure.

2.4. Serum ceruloplasmin

Serum ceruloplasmin concentrations were determined by its *p*-phenylenediamine (PPD) oxidase activity [11]. The oxidation of PPD at pH 5.4 yields a product that is readily detectable colorimetrically at 530 nm. The rate of product formation is proportional to the concentration of ceruloplasmin.

2.5. Cu,Zn-Superoxide dismutase

Total superoxide dismutase (SOD) activity was determined by a NBT assay according to Spitz and Oberley [12]. Mn-SOD activity was assayed by adding NaCN (5 μM) to the assay buffer, and the Cu,Zn-SOD was calculated by subtracting the Mn-SOD activity from the total SOD activity.

2.6. Metallothionein

Total heart tissue MT concentrations were determined by a cadmium–hemoglobin affinity assay [13] as described previously [8].

2.7. Statistical analysis

Data were analyzed initially by two-way ANOVA. Scheffe's *F*-test was employed for further determination of the significance of differences. Differences between MT-TG and WT mice were considered significant at $P<.05$. The data are presented as mean±S.D. values from the indicated number of animals for each treatment.

3. Results

The data presented in Table 1 summarize the effect of dietary copper deficiency on several parameters after the mice fed CuD diet for 5 weeks. Serum ceruloplasmin concentrations were significantly decreased in both MT-TG and WT mice receiving CuD diet for 5 weeks. Hepatic Cu,Zn-SOD, not Mn-SOD activities, was also depressed in these animals. There were no significant differences in these biochemical changes induced by copper deficiency between MT-TG and WT mice. Cardiac hypertrophy, as

Table 1
Changes in serum ceruloplasmin and hepatic Cu,Zn-SOD and Mn-SOD activities and heart hypertrophy in WT and MT-TG mice fed CuA and CuD diets for 5 weeks

	Ceruloplasmin (μg/ml)	Cu,Zn-SOD (U/mg protein)	Mn-SOD (U/mg protein)	HW/BW (mg/g)
WT/CuA	109.3±14.3	150.9±8.7	4.9±1.6	5.2±0.6
WT/CuD	23.7±9.6*	88.5±12.6*	4.4±1.1	12.1±3.6*
MT/CuA	112.5±9.6	145.1±11.1	4.9±1.8	5.4±0.2
MT/CuD	31.0±7.9*	90.5±9.2*	4.0±1.4	7.8±1.6*,**

HW/BW indicates heart weight (mg)/body weight (g).
Data are expressed as mean±S.D. ($n=6$)
* Significantly different from controls (WT/CuA) ($P<.05$).
** Significantly different from WT/CuD group ($P<.05$).

measured by the ratio of heart weight to body weight, was observed in both MT-TG and WT mice fed CuD diet for 5 weeks. However, there was a significant inhibition in the copper deficiency-induced heart hypertrophy in the MT-TG mice (Table 1).

Mineral concentrations in MT-TG mouse heart in comparison to those in WT mice were analyzed. These minerals include copper, zinc, iron, calcium, potassium, sodium, magnesium, manganese and phosphorus. The minerals with their concentration changes in the heart of MT-TG mice and alterations by dietary copper restriction included copper, zinc and iron. Others remained the same between MT-TG and WT mice (data not shown). A foremost change in the mineral status was that total zinc concentrations in the heart were increased about 2.5-folds in the MT-TG mice (Table 2), and this high level was not affected by dietary copper restriction during early feeding. There was a significant decrease in zinc concentrations in the heart of MT-TG mice fed CuD diet relative to those fed CuA diet after 5 weeks. A significant increase in total copper concentrations in the MT-TG mouse heart was also observed. However, dietary copper restriction decreased the copper concentration to the same level found in the WT mice after feeding these animals with CuD diet for 3 weeks (Table 2). Iron concentrations in the heart of MT-TG mice fed CuA diet were stable and lower (not statistically significant) relative to WT mice during the feeding experiment. In contract, iron concentrations increased in the heart of both WT and MT-TG mice fed CuD diet for 4 weeks and remained higher in the MT-TG mouse heart after fed CuD diet for 5 weeks.

Table 2
Mineral concentrations in the heart of WT and MT-TG mice fed CuA and CuD diets for the time indicated

	Week 3	Week 4	Week 5
Zinc concentration (μg/g tissue)			
WT/CuA	72.5±3.8	70.2±7.3	88.9±25.6
WT/CuD	85.6±6.8	70.4±1.3	69.8±12.4
MT/CuA	173.1±6.5*	172.6±10.3*	190.3± 9.3*
MT/CuD	181.2±14.0*	187.6±4.3*	165.6±10.1*,**
Copper concentration (μg/g tissue)			
WT/CuA	21.9±2.1	23.9±0.7	25.1±2.7
WT/CuD	15.8±0.8*	9.7±1.3*	7.1±3.3*
MT/CuA	36.8±1.7*	35.2±1.9*	35.8±0.8*
MT/CuD	15.9±4.7*,**	8.4±0.7*,**	5.1±0.5*,**
Iron concentration (μg/g tissue)			
WT/CuA	174.2±51.1	195.1±26.2	198.3±50.3
WT/CuD	173.1±21.4	266.6±17.1*	203.5±13.0
MT/CuA	153.1±30.5	179.5±34.6	183.8±20.8
MT/CuD	159.9±62.4	233.9±8.5*	227.7±26.3*

Data are expressed as mean±S.D. ($n=6$).
* Significantly different from controls (WT/CuA) ($P<.05$).
** Significantly different from MT/CuA controls ($P<.05$).

Table 3
MT concentrations in the heart of WT and MT-TG mice fed CuA or CuD diets for 5 weeks

	MT (μg/g protein)
WT/CuA	4.9±0.7
WT/CuD	5.1±1.7
MT/CuA	127.7±6.5*
MT/CuD	116.4±4.1*,**

Data are expressed as mean±S.D. ($n=6$).
* Significantly different from controls (WT/CuA) ($P<.05$).
** Significantly different from MT/CuA controls ($P<.05$).

As shown in Table 3, MT concentrations in the heart of MT-TG mice fed CuA diet were about 26-folds higher than that in the WT mice. Dietary copper restriction did not change MT concentrations in the WT mouse heart, but decreased MT concentrations in the heart of MT-TG mice, being about 22-folds higher than those of WT mice.

4. Discussion

The results obtained from this study provide important information regarding the effect of MT overexpression on copper and zinc status in the heart and the subsequent response to dietary copper restriction. Although MT elevation caused an increase in copper concentrations in the heart, the inhibitory effect on copper deficiency-induced heart hypertrophy in the MT-TG mice unlikely resulted from the copper elevation. Upon dietary copper deficiency, copper depletion in the heart reached the same low level between the MT-TG and WT mice, suggesting MT elevation did not preserve copper pool under the dietary deficient condition. On the other hand, zinc concentrations in the heart were significantly elevated, and the extent of elevation was much more than that of copper. Many studies have demonstrated the critical role of MT in regulation of zinc homeostasis [14–16]. In particular, under the condition of redox potential changes such as oxidative stress, zinc is released from MT to perform its regulatory function of cellular protection against oxidative stress [14–16]. Since oxidative stress is involved in copper deficiency-induced heart hypertrophy [17–20], the increased availability of zinc under oxidative stress conditions in the MT-TG mouse heart is most likely

involved in the inhibition of copper deficiency-induced heart hypertrophy.

Mobilization of zinc from MT by an oxidative reaction may either constitute a general pathway by which zinc is distributed in the cell or it may be restricted to conditions of stress where zinc is needed in antioxidant defense systems [15,16]. Zinc released from MT is subsequently taken up by plasma membranes, where zinc stabilizes the membrane and prevents membrane lipid oxidative damage [21,22]. In addition, released zinc may suppress lipid peroxidation by affecting many different cellular functions, such as decreasing iron uptake and inhibiting NADPH-cytochrome *c* reductase [23].

If oxidative stress triggers zinc release from MT and the cardiac protection by MT against oxidative injury is mediated by the released zinc, a dynamic change in the level of zinc and its binding to MT during oxidative stress condition would occur. In conjunction with zinc release under oxidative stress, MT would become oxidized and the total concentrations of MT would be decreased due to the fact that metal binding makes MT resistant to microsomal degradation [24]. The results obtained here indeed showed a decrease in MT concentrations in the MT-TG mouse hearts after feeding CuD diet for 5 weeks. This decrease was accompanied by the same extent of decrease in zinc concentrations, suggesting the coordinating roles of zinc and MT in myocardial protection against oxidative stress induced by dietary copper deficiency.

Under the same oxidative stress condition, copper would also be released from MT in the MT-overexpressing mouse heart. However, the increase in copper concentrations due to MT elevation was much less than that of zinc; 50% increase in copper concentrations vs. 2.5-fold increase in zinc concentrations. This increase in copper concentrations did not appear to be able to compensate for the depletion of copper concentrations in the heart due to dietary copper restriction, as evidenced by the fact that dietary copper deficiency caused the same depletion in copper concentrations between MT-TG and WT mice. Therefore, the inhibition of copper deficiency-induced heart hypertrophy in the MT-TG mice would not result from the elevation of copper concentrations.

Dietary copper deficiency caused an increase in iron concentrations in the heart. Since iron has been shown to be importantly involved in oxidative stress [25–27], the elevation of iron in the heart may be related to copper deficiency-induced oxidative stress and heart hypertrophy. However, the present results would suggest that the elevation of iron concentrations in the heart may not be responsible for the heart hypertrophy. Dietary copper deficiency increased iron concentrations in both WT and MT-TG mouse hearts, but the elevation in the MT-TG mouse hearts lasted longer, although the reason is unknown. However, heart hypertrophy was inhibited in the MT-TG mice.

This study thus demonstrates that MT elevation in the heart causes a significant increase in both copper and zinc concentrations, but MT inhibition of dietary copper deficiency-induced heart hypertrophy is likely related to the elevation of zinc concentrations.

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