Effects of Zinc on the Induction of Metallothionein Isoforms in Hippocampus in Stress Rats

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Metallothioneins (MTs) are involved in the cellular metabolism of zinc and in cytoprotection against stress factors. Hippocampus plays a specific role in the body's response to stressors. The present study was conducted to evaluate the effects of zinc on the expression of metallothionein isoforms in the hippocampus of stress rats. The animal model of psychologic stress was developed by restraint for 4 weeks. Wistar rats were randomly assigned to 6 groups: control group, zinc-deficient group, zinc-supplemented group, and the corresponding 3 stress groups. Three separate diets of different zinc contents (1.73 ppm, 17.7 ppm, and 41.4 ppm, respectively) were used in this study. Compared with the control group, the stress groups had higher inductions of MTs and MT-1 and MT-3 mRNA in hippocampus. On the one hand, the expressions of MTs and their mRNAs in hippocampus were downregulated in the zinc-deficient group; however, their expressions were evidently enhanced in the stress zinc-deficient group. MT induction in the zinc-supplemented group was increased. Furthermore, the stress zinc-supplemented group had a more significant yield of MTs and their mRNAs. In addition, the levels of plasma cortisol, interleukin-6 (IL-6), IL-1, and nitric oxide (NO) were increased clearly in the zinc-deficient group and the stress groups. The results suggest that zinc deficiency may decrease and zinc supplementation may increase the expressions of MTs and their mRNAs in hippocampus; moreover, stress can increase their expressions dramatically. The impairment of stress on the body may be involved with the nutrition status of zinc, and zinc deficiency can lower the body's adaptability to stress. Exp Biol Med 231:1564–1568, 2006

Key words: restraint stress; metallothioneins; zinc; cortisol

Introduction

As a structural and/or functional component of numerous metalloenzymes and metalloproteins, zinc can affect many aspects of cellular metabolism, including physiologic processes such as immune function, antioxidant defense, growth, and development (1–3). Therefore, an adequate supply of dietary zinc and the maintenance of zinc homeostasis are crucial for the normal functioning of these systems. At the cellular level, metallothioneins (MTs) may be central to the homeostatic regulation of zinc metabolism (4).

MTs are a family of small, cysteine-rich, metal-binding proteins present in almost all forms of life. Genomes of higher organisms contain multiple MT genes, which encode different MT isoforms. All mammals express at least 4 types of MT: MT-1, MT-2, MT-3, and MT-4 (5, 6). MT-1 and MT-2 are expressed in almost all tissues, whereas MT-3 and MT-4 are tissue specific. MT-3, also known as growth inhibitory factor (GIF), is brain-specific MT that is expressed mainly in the hippocampus, amygdale, and cortex (7). It has been proposed that MTs play an important role in zinc homeostasis by controlling cellular zinc uptake, distribution, storage, and release. Furthermore, zinc can control MT gene expression by interacting with metal-sensitive transcription factors such as MTF-1 (8), and free zinc induces the accumulation of large amounts of zinc-containing MTs. A number of studies have suggested that zinc status affects MT concentration and its mRNA synthesis in the various tissues of growing and adult rats and mice (9, 10).

Since little attention has been paid to the putative regulatory role that zinc plays in the expressions of MT isoforms in animals exposed to psychologic stress, this study was conducted to explore how MTs respond in psychologically stress animals with different nutritional zinc status.

Materials and Methods

Animals. Wistar rats weighing 140–160 g were single housed in cages with access to food and water. Temperature conditions in the animal facility were maintained at 23°C±1°C. Animals were maintained on a 12:12-hr light:

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dark cycle. After acclimating to laboratory conditions, the rats were divided at random into 6 groups of 10 animals each: control group, zinc-deficient group, zinc-supplemented group, and their corresponding stress groups (stress control group, stress zinc-deficient group, and stress zinc-supplemented group). The rats received humane care throughout the experiment according to the guidelines of the National Institute for Environmental Studies. All efforts were made to minimize both the number of animals used and their suffering.

Experimental Diets. Three separate diets of different zinc contents were used in this study. Zinc-deficient and stress zinc-deficient rats were given a zinc-deficient diet (1.73 ppm); zinc-supplemented and stress zinc-supplemented rats (the addition of zinc in the form of $ZnSO_4$) were given zinc-supplemented diet (41.4 ppm); other groups were given normal zinc content diets (17.7 ppm). The above diets were fed for 4 weeks.

Stress Model. Iron cylinder tubes were used to induce restraint stress. The tubes were 8.5 inches long and 2.0 inches in diameter. Each tube was covered on one end with a Plexiglas covering, which had a small air hole (0.2 inches in diameter) in it. These tubes fit closely to the body size of the animals and inhibited movement. Each rat received restraint for 6 hrs every day.

Determination of Plasma Levels of Zinc, Interleukin-6 (IL-6), IL-1, Cortisol, and Nitric Oxide (NO). The zinc analysis was carried out using atomic absorption spectrophotometer (Hitachi, Inc., Tokyo, Japan) after plasma was digested by 1 *M* hydrochloric acid. Plasma levels of IL-6 and IL-1 were quantified with TPI Rat IL-6 (IL-1) ELISA kit (TPI Inc., Lynnwood, WA) according to the manufacturer's instructions. Plasma cortisol concentration was measured by Cortisol Radioimmunoassay kit (Beifang Biotech, Beijing, China) according to the manufacturer's instructions. Plasma levels of NO were measured by nitrate reductase kit (Jiancheng Biotech, Nanjing, China) according to the manufacturer's instructions.

Western Immunoblots. MT protein in rat hippocampus was analyzed by Western blotting with immunostaining (11). Briefly, protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel analysis (SDS-PAGE), and proteins were electrotransferred onto nitrocellulose membrane in Tris-glycine buffer. The membrane was blocked with 5% instant milk in Tris-buffered saline (TBS), pH 7.4 (20 m*M* Tris, pH 7.4; and 150 m*M* NaCl), washed with TBS, and incubated with antiserum in TBS. After incubation with the primary antibody (multiclonal antibodies to MTs, Santa Cruz Biotechnology, Santa Cruz, CA), the blot was washed with TBS. Once washed, the blot was incubated for 4 hrs at room temperature with biotinylated goat anti-rabbit secondary antibody. Then the blot was washed three times with TBS plus 0.05% Tween-20 for 10 mins, followed by one wash with TBS for 10 mins. After incubation with luminal for 1 min, the membrane was put on sensitizing film for exposure in a dark closet. Then the bands interacting with the primary antibody showed positive blotting after the developing and fixing procedure.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from hippocampus by a Uniq-10 column total RNA preparation kit (Sangon, Shanghai, China). Purity was confirmed by A260/A280 ratio. The expressions of MT-1 mRNA and MT-3 mRNA were determined by semiquantitative RT-PCR. Gene-specific primers of MT-1 and MT-3 (MT-1: forward, 5′-aca ccg ttg ctc cag att cac-3′; reverse, 3′-cgg agc ctg ttc acg tg-5′; MT-3: forward, 5′-gtt cct gca cct gct cgg ac-3′; reverse, 3′-cgg ata cac tta tca cga cgc-5′) were used with the following schedule: 50°C 30 mins for RT; 94°C 2 mins for deactivation of RTase; 30 secs at 94°C, 30 secs at 60°C, and 90 secs at 72°C for 35 cycles; 7 mins at 72°C for extension. To ensure that equal amounts of reverse transcribed RNA were added to the PCR reaction, a parallel amplification of glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) mRNA was performed as an internal reference (GAPDH gene-specific primers: forward, 5′-gcc atc aac gac ccc ttc at-3′; reverse, 3′-tct tcc acc act tcg tcc gc-5′). The ratio of MT-1 (MT-3) mRNA/GAPDH mRNA intensity was used to evaluate the relative levels of MT mRNA induction.

Statistical Analysis. Results are expressed as means ± SEM. Differences among the groups were determined by two-way analysis of variance. Multiple comparisons to a specific control were assessed by use of least significant difference test. Significance was set at $P < 0.05$.

Results

Body Weight Change. The zinc-deficient rats had growth retardation, as evidenced by significantly lower body weights (Table 1). Weights of control rats increased by 17%, 22.7%, 13.6%, and 10.8% at the end of 1, 2, 3, and 4 weeks, respectively, whereas the corresponding percentages in zinc-deficient rats were 8.8%, 14.9%, 16.3%, and 11%, respectively. Zinc-deficient rats also developed dermatitis and alopecia during the experimental period, suggesting zinc deficiency. Furthermore, restraint stress aggravated rats' growth retardation.

Concentrations of Plasma Cortisol, Zinc, IL-6, IL-1, and NO. The basal plasma zinc concentration was significantly lower in zinc-deficient rats, whereas it increased in zinc-supplemented rats (Table 2). Compared with the control group, stress control, zinc-deficient, and stress zinc-deficient groups had upregulated levels of plasma cortisol ($P < 0.05$). The levels of IL-6 and NO increased in all groups of rats except the control group, and stress zinc-deficient rats had even more significant changes. The plasma IL-1 level only increased in stress control and stress zinc-deficient groups, in contrast to that of the control group.

Expressions of MT-1 mRNA and MT-3 mRNA. Restraint stress induced the increases of MT-1 mRNA and MT-3 mRNA in hippocampus (Figs. 1 and 2).

Table 1. The Changes in Body Weight Gain of Rats[a]

Group	Initial	1 Week	2 Weeks	3 Weeks	4 Weeks
Control	129.6 ± 9.08	151.8 ± 12.45	186.3 ± 9.06	211.7 ± 14.13	234.6 ± 18.41
Stress control	129.8 ± 7.03	143.7 ± 8.92[b]	179.6 ± 12.75	201.5 ± 8.24[b]	224.3 ± 11.34[b]
Zinc-deficient	129.3 ± 7.24	140.7 ± 12.30[b]	161.7 ± 9.82[b]	188.1 ± 13.84[b]	208.9 ± 12.48[b]
Stress zinc-deficient	129.5 ± 7.07	141.7 ± 7.59[b]	149.0 ± 5.65[b,c]	163.5 ± 8.26[b,c]	188.4 ± 12.46[b,c]
Zinc-supplemented	129.6 ± 6.85	149.5 ± 5.42	186.9 ± 9.80	215.6 ± 12.07	239.1 ± 13.54[b]
Stress zinc-supplemented	129.6 ± 7.75	147.5 ± 9.66	184.1 ± 12.67	209.2 ± 12.46[c]	234.6 ± 12.45[c]

[a] The rats were divided into 6 groups ($n = 10$). The control and stress control groups were fed normal zinc content diet (17.7 ppm). The zinc-deficient and stress zinc-deficient groups were given zinc-deficient diet (1.73 ppm). The zinc-supplemented and stress zinc-supplemented groups were given zinc-supplemented diet (41.4 ppm). All stress groups received restraint for 4 weeks. At the end of 1 week, 2 weeks, 3 weeks, and 4 weeks, rat body weights (in grams) were measured. Results are means ± SEM determined by two-way analysis of variance.
[b] $P < 0.05$ versus control group.
[c] $P < 0.05$ versus corresponding nonstress group.

Although zinc-deficient rats had lower levels of MT mRNAs, stress also induced their expressions, which were smaller than other stress rats. Stress zinc-supplemented rats had the largest induction of MT mRNAs among all the stress rats.

MT Production. The basal levels of MTs were lower in zinc-deficient rats than in control rats. In accordance with expressions of MT-1 mRNA and MT-3 mRNA, MT production in the hippocampus was increased in all stress group rats (Fig. 3). Although MT production had a tendency to decline in zinc-deficient rats and increase in zinc-supplemented rats, there were no differences among control, zinc-deficient, and zinc-supplemented groups.

Discussion

Many studies infer that endogenic glucocordicoids (GCs) play an important role in the pathologic impairments induced by stress (12, 13). As an essential stress hormone, GC levels can show a body's stress intensity. The present study demonstrated that plasma levels of cortisol were increased dramatically in stress rats with growth retardation. This suggested that an animal model of psychologic stress was developed successfully. In zinc-deficient rats, plasma zinc concentrations decreased evidently, and their food intakes and body weight gains diminished remarkably during the experimental period, suggesting zinc deficiency. Furthermore, plasma levels of cortisol, IL-6, and IL-1 also changed obviously in zinc-deficient rats.

MTs are involved in the regulation of cellular metabolism of zinc, the detoxification of toxic metals like cadmium and copper, and the protection of cells against various stressors (14–16). The isoforms MT-1/2 are important antioxidants and tissue protecting factors that may also be equally important for normal brain physiology, since significant upregulations have been observed in such human pathologies as Alzheimer and Pick diseases and amyotrophic lateral sclerosis, as well as animals subjected to stress, lipopolysaccharide (LPS), or brain damage (17–20). Furthermore, MT-2 modulation can enhance the survival rates of dopaminergic neurons exposed to 6-hydroxyl dopamine and protect hippocampal neurons against neurotoxicity induced by β-amyloid (21). MT-3 also is an essential neural protecting factor, and it has an intensive relationship with zinc. MT-3 binds zinc with specificity, and expression of MT-3 can selectively increase the capacity of neurons to contain zinc ions (22). By regulating zinc's uptake, storage, and transmission, MT-3 may affect the biologic synthesis and activity of zinc-binding proteins, the activity of enzymes dependent on zinc, and the activities of

Table 2. The Changes of Plasma Cortisol, Zinc, IL-6, IL-1, and NO in Rats[a]

Group	Cortisol (ng/ml)	Zinc (mg/l)	IL-6 (ng/ml)	IL-1 (ng/ml)	NO (ng/ml)
Control	42.08 ± 24.29	1.25 ± 0.25	10.25 ± 3.57	8.34 ± 3.65	21.54 ± 8.92
Stress control	64.67 ± 31.36[b]	1.24 ± 0.15	12.46 ± 4.19[b]	9.26 ± 5.32[b]	24.87 ± 9.51[b]
Zinc-deficient	79.22 ± 23.58[b]	0.83 ± 0.35[b]	16.84 ± 8.44[b]	9.06 ± 5.06	39.26 ± 9.47[b]
Stress zinc-deficient	104.47 ± 34.28[b,c]	0.78 ± 0.42[b]	24.17 ± 10.63[b,c]	10.57 ± 5.48[b,c]	42.19 ± 11.50[b,c]
Zinc-supplemented	28.39 ± 19.29	1.73 ± 0.25[b]	12.47 ± 5.74[b]	7.84 ± 3.41	24.68 ± 9.27[b]
Stress zinc-supplemented	37.15 ± 18.97	1.79 ± 0.26[b]	13.56 ± 7.42[b]	8.61 ± 4.09	26.19 ± 10.43[b]

[a] The rats were divided into 6 groups ($n = 10$). The control and stress control groups were fed normal zinc content diet (17.7 ppm). The zinc-deficient and stress zinc-deficient groups were given zinc-deficient diet (1.73 ppm). The zinc-supplemented and stress zinc-supplemented groups were given zinc-supplemented diet (41.4 ppm). All stress groups received restraint for 4 weeks. At the end of experiment, plasma was separated for examination of cortisol, zinc, IL-6, IL-1, and NO. Results (in grams) are means ± SEM determined by two-way analysis of variance.
[b] $P < 0.05$ versus control group.
[c] $P < 0.05$ versus corresponding nonstress group.

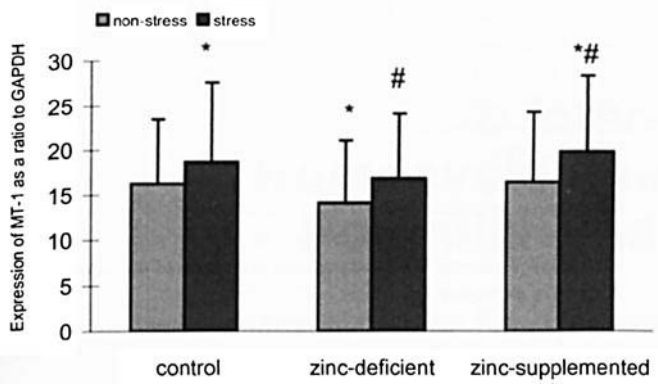

Figure 1. Hippocampal MT-1 mRNA expression as assessed by RT-PCR. The rats were divided into 6 groups ($n = 10$). The control and stress control groups were fed normal zinc content diet (17.7 ppm). The zinc-deficient and stress zinc-deficient groups were given zinc-deficient diet (1.73 ppm). The zinc-supplemented and stress zinc-supplemented groups were given zinc-supplemented diet (41.4 ppm). At the end of experiment, hippocampus was separated for examination of MT-1 mRNA. Results are means±SEM determined by two-way analysis of variance. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus corresponding nonstress group.

some transcriptional factors. Zinc-MT modulation can significantly decrease the production of IL-6 and tumor necrosis factor-α (TNF-α) in the central neural system (23).

MT expression can be regulated by many factors, such as cytokines, stress, hormones, metals, oxidants, and toxic agents (24, 25). IL-6 and TNF-α are significant inducers of MTs in the brain (26–28). The upregulation of IL-6 in the central neural system by transgenic methods can regulate the expression of many genes, including the MT family (6). There is diminished body weight growth and impaired Rotarod test in mice treated with IL-6 transgenic high expression and MT deficiency. Moreover, macrophages and T

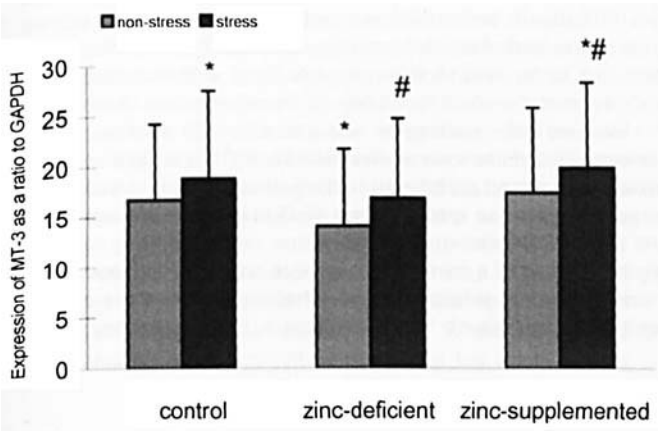

Figure 2. Hippocampal MT-3 mRNA expression as assessed by RT-PCR. The rats were divided into 6 groups ($n = 10$). The control and stress control groups were fed normal zinc content diet (17.7 ppm). The zinc-deficient and stress zinc-deficient groups were given zinc-deficient diet (1.73 ppm). The zinc-supplemented and stress zinc-supplemented groups were given zinc-supplemented diet (41.4 ppm). At the end of experiment the hippocampus was separated for examination of MT-3 mRNA. Results are means ± SEM determined by two-way analysis of variance. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus corresponding nonstress group.

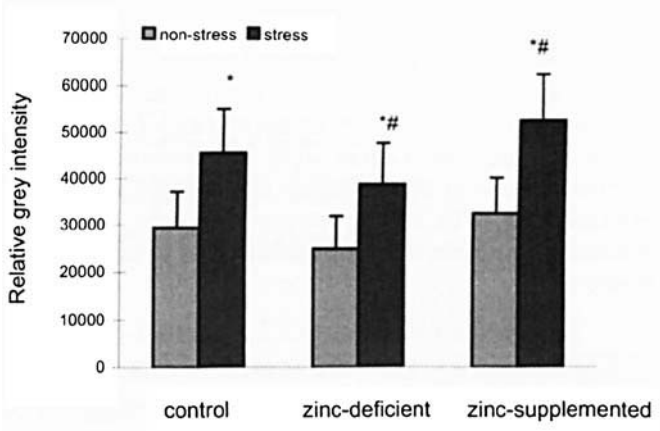

Figure 3. Hippocampal MT production as assessed by Western blotting. The rats were divided into 6 groups ($n = 10$). The control and stress control groups were fed normal zinc content diet (17.7 ppm). The zinc-deficient and stress zinc-deficient groups were given zinc-deficient diet (1.73 ppm). The zinc-supplemented and stress zinc-supplemented groups were given zinc-supplemented diet (41.4 ppm). At the end of experiment the hippocampus was separated for examination of MT proteins. Results are means ± SEM determined by two-way analysis of variance. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus corresponding nonstress group.

lymphocytes in the central neural system were aggregated and activated in those mice, and the levels of cytokines such as IL-6, IL-1, and TNF-α also were increased. Increased expression of MT-1/2 can antagonize this kind of brain injury (29).

In our study, the expressions of MTs, MT-1 mRNA, and MT-3 mRNA in hippocampus were increased dramatically in rats exposed to 4 weeks of restraint. Moreover, MT production was downregulated in zinc-deficient rats, whereas it was upregulated in stress zinc-deficient rats. Although stress zinc-deficient rats had the highest levels of plasma IL-6, IL-1, and cortisol, their production of MTs was not the largest among all 3 stress groups. The results suggest that the nutrition status of zinc may play an important role in the induction of MTs in hippocampus, and that zinc deficiency can lower the body's adaptability to stress.

In addition, the changes of NO also were examined, and its plasma levels were increased evidently in stress rats and zinc-deficient rats. NO is an important regulator of signal transmission in cells. There is an intense association among NO, zinc, and MTs. Studies show that NO produced from donor compounds can induce dissociation of zinc from various metalloproteins such as alcohol dehydrogenase, MTs, and zinc-finger transcription factors (30). The released zinc ions would then modify gene expression, and, indeed, this appears to occur with the MT gene. Thus, the interactions between NO and MTs could have important roles in zinc homeostasis and, potentially, in gene regulation (30). Furthermore, MTs and zinc can inhibit the production of NO by inhibiting the expression of induced NO synthase (iNOS) (31, 32). Excess NO was produced mainly by iNOS, thereby bringing about pathologic damages to some tissues and cells. Thus, by regulating NO production MTs and zinc

may modulate NO at a rational level. In the present study, it was observed that NO levels were in accordance with the expression of MTs, which may be a result of the interaction described above.

In summary, the current study demonstrates that the nutritional status of zinc may play an important role in the expression of MTs in hippocampus in stress rats. Zinc deficiency may lower the body's adaptability to psychologic stress.

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