Modulated Responses to Restraint Stress and Inflammation in Metallothionein-Null Mice

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The present study was designed to investigate the effects of restraint stress and inflammation on levels of serum zinc and corticosterone in metallothionein-null ($MT^{-/-}$) mice with respect to a modulating role of MT in stress responses. In wild-type ($MT^{+/+}$) mice, serum zinc concentration decreased after injection of lipopolysaccharide (LPS), but increased with exposure to restraint. In LPS-treated mice, serum zinc levels were higher in $MT^{-/-}$ mice than in $MT^{+/+}$ mice. On the other hand, after exposure to restraint stress, serum corticosterone levels increased in both mice, but the increase rate was higher in $MT^{+/+}$ mice than in $MT^{-/-}$ mice. In addition, injection of LPS decreased serum corticosterone in $MT^{+/+}$ mice, but there was no change in $MT^{-/-}$ mice. These findings suggest that MT may modify the zinc metabolism and stress responses in inflammatory and restraint stress.

Key words —— metallothionein, restraint stress, zinc, lipopolysaccharide, stress response

INTRODUCTION

Much attention has recently been paid to lifestyle-related diseases in many industrialized nations. These diseases are usually due to excessive energy intake, a general lack of exercise, and stress in modern life. Stress is generally defined as a state of altered homeostasis caused by an external or internal stimulus, which has been shown to be restored to the control level by a variety of adaptation mechanisms. It has been shown that mental and physiological stresses induce and progress lifestyle-related diseases.[1, 2]

Metallothionein (MT), a low-molecular-weight, cysteine-rich metal-binding protein, is induced under various stressful conditions, including exposure to heavy metals, glucocorticoids, cytokines, fasting stress, and restraint stress.[3–6] MT is thought to be an acute phase protein[7] involved in the cellular metabolism of zinc and in cytoprotection against stress factors. The major metal bound to MT in tissues is zinc.

MT-null ($MT^{-/-}$) mice have been established[8, 9] and are widely used to investigate the physiological functions of MT, as follows. As compared with wild type control mice, $MT^{-/-}$ mice are vulnerable to oxidative stress such as acetoaminophen[10] or cadmium nephrotoxicity,[11] and showed higher sensitivity in DNA damage by restraint stress[12] or in immune responses with elevated nuclear factor-κB (NF-κB) activation.[13] The mice also showed a poorer rate of learning.[14]

Zinc, an essential trace element, plays a unique role as a catalytic and structural cofactor for hundreds of zinc-dependent metallo-enzymes and transcription factors, and is needed for normal growth and development of the body.[15] Zinc deficiency, therefore, can give rise to gross morphological, biochemical, and functional abnormalities in mammals.[16] The concentration of zinc in serum varies in a variety of conditions such as stress, zinc deficiency in food or water, stage of development, and inflammation.

In the present study using $MT^{-/-}$ mice, we have investigated whether MT-inducibility contributes to the regulation of serum zinc and corticosterone in inflammatory stress and non-physiological stress.

MATERIALS AND METHODS

Animals —— $MT^{-/-}$ mice (129/Sv-*MT1MT2*$^{tml\ bri}$), which were developed by Masters *et al.*,[9] and 129/Sv (wild type) mice ($MT^{+/+}$ mice) were originally purchased from Jackson Laboratory (Bar

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Harbor, ME, U.S.A.) and reproduced by serial breeding. Mice were maintained at 23 ± 1.5°C with a 12-hr light-dark cycle (lights on from 8:00 to 20:00) with continuous access to water and a commercial laboratory chow diet (Type MF, Oriental Yeast, Tokyo, Japan). All chemicals were of reagent grade.

Treatment of Mice —— In order to compare stress responses involving the production of glucocorticoid hormone, which is affected by various hormones such as growth hormone or sex hormones, mice with a stable growth phase without sex cycle, such as 5–7 month-old male mice, were selected.

Male $MT^{+/+}$ and $MT^{-/-}$ mice (5–7 months old) were subjected to restraint stress for 24 hr using a metallic wire, which reproducibly induced MT (as an indicator of stress loading) in liver of $MT^{+/+}$ mice in a preliminary experiment, or were subcutaneously inoculated with lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of $2 \mu g/g$ of body weight, which is known to induce systemic inflammatory responses in liver. Blood samples were obtained via a carotid artery under pentobarbital anesthesia after the 24 hr restraint treatment or 24 hr after the LPS-injection in order to adjust the total stress-loading period in the two treatments, and the mice were subsequently killed by cervical dislocation in order to remove the liver. Blood was left for 2 hr at 37°C to separate the serum from the clot. Sera and liver tissues were stored at −80°C until use. All experimental procedures were approved by the Animal Care and Use Committee of Tokushima Bunri University and con-

formed to the guidelines established by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Determination of Serum Corticosterone, Interleukin-6 (IL6) and Zinc —— Serum corticosterone level, which is thought to be an indicator of stress response involving activation of the pituitary-adrenal axis, and serum IL6 level, an indicator of LPS-induced inflammation, were estimated using a mouse corticosterone analysis kit (Assay Desibns, Inc., Ann Arbor, MI, U.S.A) and an interleukin-6 ELISA kit (EM2-IL6, Pierce Chemical Co.,U.S.A.), respectively, according to the manufacturer's instruction. Serum zinc levels were determined using an atomic absorption frame spectrometer (Z-8200, Hitachi Co., Ltd., Tokyo, Japan).

Measurement of Hepatic MT and Zinc —— Hepatic MT levels were estimated using a Cd-hem method described previously.[17, 18] Zinc content in liver homogenate was determined using an atomic absorption frame spectrometer (Z-8200, Hitachi Co., Ltd.) as described above, followed by wet-ashing using nitric and sulfuric acids.

Statistical Analysis —— Statistical significance was determined using the two-tailed Student's t-test.

RESULTS

Figure 1 shows the deficient phenotype in MT induction of $MT^{-/-}$ mice. In $MT^{+/+}$ mice, both the LPS-injection and restraint resulted in an increase of hepatic MT protein, while in the $MT^{-/-}$

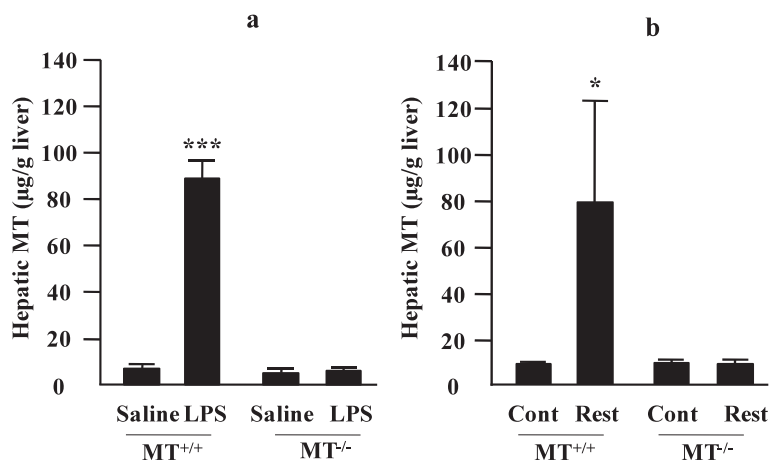**Fig. 1.** Effects of LPS and Restraint on Hepatic MT Protein in $MT^{+/+}$ and $MT^{-/-}$ Mice

Mice were injected with LPS (a) and exposed to the restraint (b), and were killed 24 hr after the LPS inoculation and termination of the restraint, respectively. Next, the liver tissues were excised and the hepatic levels of MT protein were determined as described in the Methods section. The columns and the bars are the means and S.D. of the mean, respectively ($n = 3$ mice). $^{*}p < 0.05$, and $^{***}p < 0.001$ vs. respective control.

mice, neither treatment altered the levels of hepatic MT. Though slight background MT protein was detected even in MT$^{-/-}$ mice, this seems to be because some proteins, other than MTI/II, showed the same characteristics as MT in the Cd-hem method, by which MT is analyzed as proteins that cannot be precipitated even when treated at 100°C and have cadmium-binding ability. Indeed, MT$^{-/-}$ mice did not show MT induction in the stress loadings (Fig. 1a and 1b). Thus, these observations confirmed that the MT$^{-/-}$ mice we generated retained the MT-deficient phenotype.[9]

Next, the effects of LPS administration and restraint stress on zinc kinetics were investigated. Twenty-four hr after the injection of LPS, the serum zinc concentration decreased slightly as compared with the saline-treated control in MT$^{+/+}$ mice, but

increased slightly in MT$^{-/-}$ mice, although not significantly (Fig. 2a). While exposure to restraint stress for 24 hr resulted in significant increases in serum zinc in both MT$^{+/+}$ mice and MT$^{-/-}$ mice as compared with their respective controls, the increase was greater in MT$^{-/-}$ mice than in MT$^{+/+}$ mice (Fig. 2b). Hepatic zinc level was increased by both the LPS-injection and the restraint only in MT$^{+/+}$ mice, whereas, in MT$^{-/-}$ mice, neither treatment brought about a change in hepatic zinc levels (Fig. 2c and 2d).

Figure 3 shows the serum levels of corticosterone and IL6. The serum corticosterone concentration was significantly increased in MT$^{+/+}$ mice, but was not changed in MT$^{-/-}$ mice, by injection of LPS (Fig. 3a). On the other hand, the restraint stress resulted in significant increases in serum corticos-
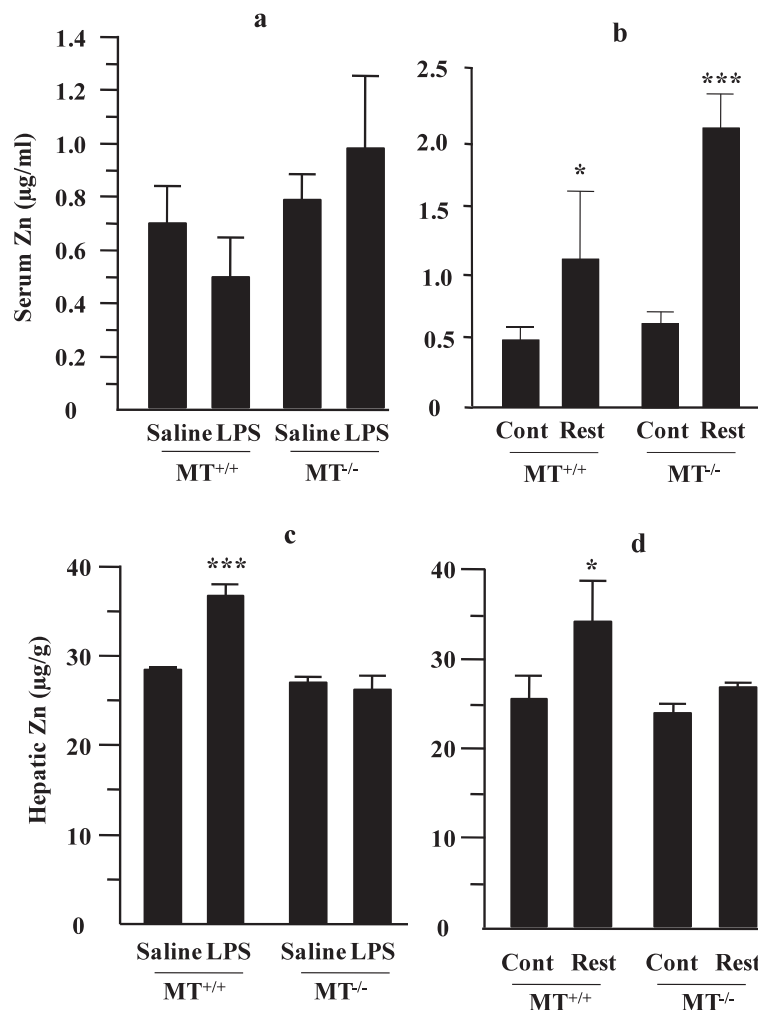

Fig. 2. Effects of LPS and Restraint on Serum and Hepatic Zinc Levels in MT$^{+/+}$ and MT$^{-/-}$ Mice

Mice were inoculated with LPS and exposed to the restraint stress, and were killed 24 hr after the LPS inoculation and termination of the restraint, respectively. Serum and liver samples were subjected to the determination of zinc as described in the Methods section. In (a) and (c), Saline, treated with saline instead of LPS. In (b) and (d), Control, non-treated mice. The columns and the bars are the means and S.D. of the mean, respectively (n = 3–6 mice). $^{*}p < 0.05$, and $^{***}p < 0.001$ $vs.$ respective control.

terone levels in both mice (Fig. 3b). The proportional increase as compared with control mice was about 2 times higher in MT$^{+/+}$ mice (5.6 times) than in MT$^{-/-}$ mice (2.9 times). After injection of LPS, IL6 concentration increased in both mice, whereas exposure to restraint stress did not change the IL6 concentration in either. These findings suggest that the restraint stress did not cause apparent inflammatory events such as local injury due to the wire-restriction.

DISCUSSION

In MT$^{+/+}$ mice, both the LPS-injection and restraint stress resulted in significant increases in serum corticosterone and hepatic MT protein (Fig. 1 and Fig. 3a, b). Only LPS-injection induced the increase in serum IL6 (Fig. 3c). Since IL6[19, 20] and corticosterone[21, 22] have been shown to induce MT, although not always synergistically or additively, the increased hepatic MT may be induced by increased corticosterone in restraint-exposed mice, and partly by increased IL6 in LPS-treated mice. Thus, the two treatments used here are qualitatively different stressors.

Since exposure to restraint stress was carried out without food intake, there is a possibility that the increase in hepatic MT in restraint-exposed mice was due to fasting. However, the restraint caused about an 8-fold increase in hepatic MT, while the 24 hr fasting alone resulted in only a 2-fold increase in a previous study,[23] indicating that most of the increased MT seems to be due to the restraint itself.
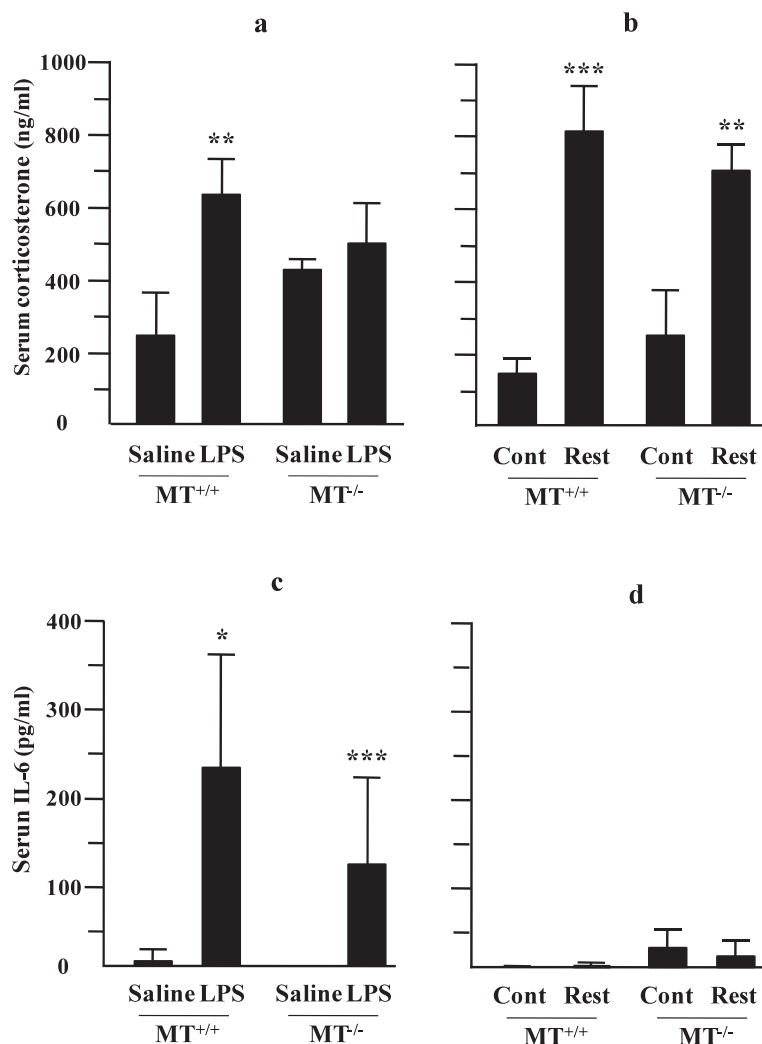

Fig. 3. Effect of LPS and Restraint on Serum Corticosterone and IL6 in MT$^{+/+}$ and MT$^{-/-}$ Mice

Serum samples of the mice in Fig. 2 were used for evaluation of serum corticosterone and IL6 as described in the Methods section. The columns and the bars are the means and S.D. of the mean, respectively ($n = 3$–6 mice). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ $vs.$ respective control.

Concerning the zinc kinetics, an increase in hepatic zinc was observed only in MT$^{+/+}$ mice by both the LPS and restraint treatments. These increases in hepatic zinc seem to be correlated, at least in part, with the increased hepatic MT proteins. If calculated from the data in Fig. 2, the increase in hepatic zinc by LPS treatment and the restraint is assumed to be 8.4 and 8.2 μg/g liver, respectively, values that are comparable to the putative maximum zinc amount (5.6 and 4.9 μg/g liver, respectively) able to saturate the newly induced MT proteins (Fig. 1) (7 Zinc molecules/thionein with mean hypothetical molecular weight of 6700). In conjunction with no change in hepatic MT in MT$^{-/-}$ mice, these findings suggest that hepatic MT induction would contribute to the increased zinc in liver and that MT could play a role in retaining zinc in liver as an MT-bound form.

LPS-injection slightly decreased the serum concentration of zinc in MT$^{+/+}$ mice, while it did not bring about any change in MT$^{-/-}$ mice (Fig. 2a). Gaetke et al.[24] showed that LPS-injection increased plasma cytokines and decreased serum zinc in humans, and that both the urinary excretion and binding ability to albumin of zinc were not changed. This agrees with our results. Although the data are not shown, 18 hr after the LPS-injection, serum zinc levels in MT$^{+/+}$ mice were significantly decreased compared with those in the saline control. This LPS-induced decrease in serum zinc was probably due to the redistribution of zinc into liver as described above and cytokine-directed internal redistribution.

The restraint significantly increased the serum zinc in both mice, although the increase was greater in MT$^{-/-}$ mice than in MT$^{+/+}$ mice (Fig. 2b). These observations suggest the restraint basically caused the influx of zinc into the blood from organs other than MT-inducing organs such as liver, because the mice were not given zinc in their food or water during the restraint. In MT$^{+/+}$ mice, this influx may have been reduced by the redistribution of zinc into liver, whereas in MT$^{-/-}$ mice, such a redistribution may not have occurred.

The basal (control) level of serum corticosterone was slightly higher in MT$^{-/-}$ mice than in MT$^{+/+}$ mice, although the difference was not significant (Fig. 3a and 3b). This may be explained as being a result of constitutive exposure to the increased oxidative stress due to the loss of MT-inducibility, by which reactive oxygen species that develop in usual cellular respiration are diminished in MT$^{+/+}$ mice.

It is noteworthy that in the LPS-treated mice, the serum corticosterone level increased in MT$^{+/+}$ mice, but was not changed in MT$^{-/-}$ mice (Fig. 3a), and the serum IL6 level increased in both mice. These findings suggest the extent of the stress response may be weaker in MT-deficient mice than in MT$^{+/+}$ mice. MT must play a role in maintaining a normal stress response. As zinc is an important metal factor for various types of transcriptional regulation, the increase in hepatic MT protein due to restraint stress may contribute to efficient tissue regeneration or destruction through the concentration of zinc into liver, which is an organ that produces many stress-responsible proteins, to generate appropriate gene expression. However, the role of MT in maintaining the stress response is not well known.

We have previously studied the induction of MT and its role in the responses to stresses originating in subcellular organelles, such as mitochondria[25–28] and endoplasmic reticulum.[29] Thus, further studies are required to clarify the role of MT as a stress response molecule in the whole body as well as subcellular organelles.

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