INTRODUCTION

The presence of excess reactive oxygen species (ROS) can cause cellular damage via oxidation of biomolecules, including DNA, proteins and polyunsaturated fatty acids, causing a variety of diseases such as cancer, Parkinson’s disease, and cardiovascular disease (1, 2). It has been shown that mental and physiological stresses induce excess ROS and worsen lifestyle-related diseases (3-5). To reduce the formation of excess ROS is important for preventing the development of diseases.

The authors have been studying the metallothionein (MT) synthesis induced by a variety of...
stresses, and the antioxidant effect of MT (6-10). MT is known as a cysteine-rich, low molecular weight and metal-binding protein (11) and has several interesting biological effects including detoxification of heavy metals, regulation of the homeostasis of essential metals, and antioxidant effects against reactive oxygen species (12-15). We previously reported that food-induced dyslipidemia in mice could be accelerated by repeated fasting, but could be suppressed by repeated restraint, and that the DNA damage in cells of various organs was increased by the repeated stress, especially in MT-null mice (9). Glutathione (GSH) is the most abundant antioxidant in the liver, and plays most significant role against toxicity of ROS including superoxide, hydrogen peroxide and hydroxyl radicals. Among ROS, hydroxyl radicals have the strongest toxicity. Although GSH concentration is greater than those of other antioxidants in the liver, MT has the strongest affinity with the hydroxyl radicals (16). MT may play a preventive role against ROS in cooperation with or instead of GSH (12, 17, 18). In our previous study, however, we could not explore the relationship between GSH and MT under repeated stress, due to the 12-h fasting before sacrifice to determine serum cholesterol levels (9). Corticosterone level which is known as an indicator of biological stress (19). In the previous paper, we also reported that the body weight of restraint mice fed high-fat diet (HFD) was markedly decreased although the total energy intake was higher than that of control mice fed standard diet (STD), suggesting that the energy metabolism system may be changed by the repeated restraint stress. In the present study, we investigated two factors, carnitine and adiponectin, in the energy metabolic system. Carnitine is an amino acid derivative which is essential for the incorporation of long-chain fatty acid into mitochondria (20-22). Adiponectin is an anti-diabetic adipokine which is specifically and abundantly produced in the white adipose tissue (23-25). We describe here the results of investigation concerning to the effects of repeated mild stress on the biological defense systems and lipid metabolic systems in mice fed dyslipidemia-inducing HFD.

EXPERIMENTAL METHODS

Animal treatments

Female adult 129/Sv wild-type and MT-null mice (Jackson Laboratory, USA; 21-24 g in body weight) were each divided into six groups. The mice assigned to the stress groups were fed HFD for 4 weeks and were subjected to fasting for 24 hours from 20:00 on every other day, three times per week (F24), or to restraint with tube for 15 min (R15), 30 min (R30) or 60 min (R60) daily on weekdays, in weeks 2 to 4 of the 4-week study period as shown in the previous paper (9). Control mice were fed STD (C1) or HFD (C2) for 4 weeks without being subjected to any stress. The HFD (Crea Japan, Osaka, Japan), specially produced for our laboratory, was based on the AIN 76 diet (26), containing 12% corn oil, 13% beef tallow, 1% cholesterol, 0.2% cholic acid, and 418.2 kcal/100 g. The STD (CE-2) containing 4.4% fat and 342.2 kcal/100 g was purchased from Crea Japan. These solid-type diets were weighed and fed adequately. Total energy intakes were calculated as the sum of diet eaten by each treatment groups during the experiment. Cages of the animals were cleaned regularly during the fasting period to prevent coprophagy. Restraint was performed using a handmade restraint device, a 50-ml plastic centrifuging tube modified for this experiment, as shown in Fig. 1. It has twelve 3-mm diameter holes for sweating, and one hole for tail-protecting, and four holes fitted two steel rings for the animal’s habitual teeth grinding and for protecting the holes for animal’s breathing. Volume of centrifuging tube was changed in response to the size of

Fig. 1 Handmade restraint device. A 50 ml-plastic centrifuging tube has twelve 3-mm diameter holes for sweating, and one hole for tail-protecting, and four holes fitted two steel rings for the animal’s habitual teeth grinding and for protecting the holes for animal’s breathing.
the animal. At the end of the treatment period, mice loaded with stress were fed HFD for approximately one day, and all the animals were fasted for 2 hours before they were sacrificed. All experimental procedures were approved by the Animal Care and Use Committee of Tokushima Bunri University and conformed to the guidelines established by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Sample preparation

At the end of the 4-week study period, blood samples were collected from the supraorbital vein of the animals under pentobarbital anesthesia. Then, the liver, pancreas and spleen were quickly removed from each animal, weighed and placed on ice. The bone marrow cells were collected from the femur and eluted with a phosphate buffered saline.

Comet assay

Aliquots of mouse organs (liver, pancreas and spleen) were homogenized. The homogenates and bone marrow eluate were subjected to the comet assay (27, 28) using the CometAssay and Silver Staining Kits (Trevigen, Gaitherburg, MD, USA) and SYBR Gold (Invitrogen, Eugene, OR, USA). The comet images obtained using an optical or a fluorescence microscope equipped with a digital camera were analyzed and were scored using a comet analysis software (CometAnalyzer, Youworks, Tokyo, Japan). The DNA damage was expressed as a ratio of the total comet length to the diameter of the head DNA from 100 cells of each organ.

Protocols for other assays

Liver and serum samples were stored at -35°C and 4°C until assay, respectively. Total GSH levels in the liver were determined with the GSH Quantification Kit (Dojindo, Kumamoto, Japan). Hepatic MT concentrations of wild-type mice were determined by the Cd-heme method (29). Serum corticosterone, adiponectin and hepatic total carnitine levels were determined with the respective quantification kits (Assay Designs, Ann Arbor, MI, USA; Otsuka Pharmaceutical, Tokyo, Japan; and KAINOS Laboratories, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SEM. Differences in means were analyzed using unpaired t-test with InStat 2.0 (GraphPad Software Inc.). Statistical significance was defined as p<0.05.

RESULTS

Body weight change

Body weights of wild-type mice of HFD control (C2) and 24-h fasted group (F24) were similarly increased and were higher than those of mice fed STD (C1) as shown in Fig. 2A. On the other hand, the increases in body weight of the restraint groups (R15, R30, R60) were clearly reduced in proportion to the intensity of stresses as shown in Fig. 2A. Similar tendencies were seen in MT-null mice, although their weight gains were slightly greater than those of wild-type mice as shown in Fig. 2B.

![Fig. 2](https://example.com/figure2.png)

Change in body weight in (A) wild-type and (B) MT-null mice fed standard diet or high-fat diet with or without fasting or restraint stress.

- C1, standard diet control; C2, high-fat diet control; F24, high-fat diet plus fasting for 24 h; R15, R30, and R60, high-fat diet plus restraint for 15, 30, and 60 min, respectively. Data are presented as mean ± SEM, n=10. * p<0.05 vs. corresponding C2 group.

Total energy intake

Total energy intakes in the wild-type and MT-null mice groups were expressed as the percentage to the total energy intake in the corresponding STD groups (Fig. 3). Relative energy intake was higher for all groups fed HFD than that for the corresponding STD group as shown in Figs. 3A and 3B. Both in wild-type and MT-null mice, total energy intake of 24-h fasting groups was similar to that of the corresponding HFD control group, whereas total energy intake of three restraint groups was slightly
reduced compared with that of the corresponding HFD control group. In general, the fat-energy intakes of all HFD groups were much higher than that of the corresponding STD group.

Liver weight

In wild-type mice, the liver weight of HFD control group was significantly higher than that of STD control group, and that of 24-h fasting group was extremely higher (ca. 1.9 fold) than that of HFD control group as shown in Fig. 4. On the other hand, the liver weights of restraint groups were slightly lower than that of the HFD control group. Similar tendency was observed in the liver weights of MT-null mice. There were no significant differences in the liver weights between wild-type mice and MT-null mice in the corresponding treatment group, although the weights of MT-null mice tended to be a little higher than those of wild-type mice.

As was previously reported (9), the other organs, pancreas and spleen, excised from the mice showed no distinct differences both in weight and shape among the treatment groups.

Hepatic concentrations of GSH and MT

Total GSH levels in the liver of mice were shown in Fig. 5. In the wild-type mice, the GSH level of HFD control group was significantly decreased compared to that of STD control group, and the GSH levels were gradually reduced as the length of restraint time increased. Similar tendency was observed in the MT-null mice, and the GSH levels
were clearly lower than those of wild-type mice.

On the other hand, MT levels in the liver of wild-type mice was markedly increased (ca. 1.6 fold of C1) by 24-h fasting, and were increased as the length of restraint time increased, as shown in Fig. 6.

![Graph showing metallothionein levels in wild-type mice fed standard diet or high-fat diet with or without fasting or restraint stress.](image)

**Fig. 6** Hepatic metallothionein levels in wild-type mice fed standard diet or high-fat diet with or without fasting or restraint stress. C1, standard diet control; C2, high-fat diet control; F24, high-fat diet plus fasting for 24 h; R15, R30, and R60, high-fat diet plus restraint for 15, 30, and 60 min, respectively. Data are presented as mean ± SEM, n=10. * p<0.05 vs. C2 group.

Serum corticosterone concentration

Corticosterones in the serum of wild-type mice were markedly increased in the restraint groups compared with STD- and HFD-control groups as shown in Fig. 7. Similar tendency was observed in the serum corticosterone levels of MT-null mice, which were slightly higher than those of wild-type mice in general.

DNA damage in the cells of mouse organs

DNA damages in the liver, pancreas, spleen and bone marrow cells of wild-type and MT-null mice were shown in Fig. 8. The increase of DNA damage was generally seen in the cells of mice in restraint groups, but not so clear in the cells of fasted mice. Specifically, in the MT-null mice, the DNA damages in almost all organ cells of restraint mice were markedly increased as shown in the figures A-D. On the other hand, in wild-type mice, DNA damages were significantly increased in the pancreas and bone marrow of the 30-min restraint group, and in the liver, spleen and bone marrow of the 60-min restraint group. In all restraint groups, the levels of DNA damage in MT-null mice were clearly greater than those in wild-type mice. Significant increases in DNA damage by the HFD and 24-h fasting stress were not observed in the present study.

Changes in hepatic carnitine and serum adiponectin levels

In the wild-type mice, hepatic total carnitine levels were not so affected by the stresses of fasting and restraint in the present study, as shown in Fig. 9. On the other hand, in the MT-null mice, the carnitine levels were markedly decreased compared with those in the wild-type mice. In the restraint mice, the carnitine levels were particularly decreased with the increase of stress intensity compared with those in STD control mice.

Serum adiponectin level was significantly decreased in HFD control group compared with STD control group and was further decreased in 24-h fasting group as shown in Fig. 10. On the other hand, the adiponectin levels in restraint groups were increased up to almost the same as that in STD control group. It is suggested that the adiponectin reduction by a factor(s) contained in HFD may be inhibited by the repeated mild restraint stress. No differences in the serum adiponectin level were observed between the two types of mice in the present study.

![Graph showing serum corticosterone levels in wild-type and MT-null mice fed standard diet or high-fat diet with or without fasting or restraint stress.](image)

**Fig. 7** Serum corticosterone levels in wild-type and MT-null mice fed standard diet or high-fat diet with or without fasting or restraint stress. C1, standard diet control; C2, high-fat diet control; F24, high-fat diet plus fasting for 24 h; R15, R30, and R60, high-fat diet plus restraint for 15, 30, and 60 min, respectively. Data are presented as mean ± SEM, n=10. * p<0.05 vs. corresponding C2 group.
DISCUSSION

It has been shown that GSH is probably the most important cellular antioxidant (17, 30). MT may act as a secondary antioxidant in a cellular protection system in the absence of GSH (18, 31). In the previous
study (9), we failed to observe the exact accumulated effect by repeated stress on the primary biological defense systems involving GSH and MT because mice were fasted for 12 hours before sacrifice to determine serum cholesterol. In the present study, however, we could investigate the defense systems in 129/Sv wild-type and MT-null mice fed standard diet or high-fat diet with or without fasting or restraint stress, by limiting the fasting time before sacrifice to 2 hours. It was clearly shown that the total amount of GSH stored in the liver was gradually decreased with the intensity of stress increased both in the wild-type and MT-null mice (Fig. 5), and that hepatic MT levels of wild-type mice were increased in response to the decline of GSH, suggesting that MT plays a preventive role as an antioxidant in corporation with decreased GSH which is a primary radical scavenger for DNA and lipid metabolic damages in murine organs induced by repeated mild stress (12, 17, 18). If MT has more potent antioxidant effect than GSH against the hydroxyl radicals which are known as highly strong reactive oxygen species interacting with DNA, it may act not only as a supplementary radical scavenger but also as an extraordinarily efficient hydroxyl radical scavenger, as stated by Thornalley and Vasák (16). The specific induction (1.6 fold) of MT in the liver of wild-type mice subjected to 24-h fasting (Fig. 6) must be a specific adaptation of liver tissue in response to the repeated severe stress. In these animals, the MT levels in the hypertrophic liver (1.9 fold in weight, Fig. 4) were increased approximately three times compared with those of STD mice. Serum corticosterone levels were markedly increased in the restraint groups compared with that of the corresponding control group as shown in Fig. 7, demonstrating that stresses were loaded well in this study.

Both in wild-type and MT-null mice, the body weight gains of mice subjected to mild restraint for 60 min/day or less and fed HFD, were markedly suppressed compared with those of mice fed STD (Fig. 2), even if the total energy intakes were higher than those of control mice fed STD (Fig. 3), suggesting that the energy metabolism system may be changed by the repeated restraint stress as previously reported (9). Then, we investigated two factors involved in the energy metabolic system, carnitine and adiponectin. It was found that hepatic total carnitine levels in MT-null mice were generally decreased compared with those in wild-type mice and were particularly decreased with the increase in intensity of restraint compared with those in STD mice as shown in Fig. 9. Carnitine is known not only as an essential factor for biological lipid metabolism, but also as an antioxidant against biological reactive oxygen species (20-22, 32). Present data suggest that the hepatic carnitine levels were reduced by HFD, and that the reduction might have been enhanced by stresses and further accelerated additionally by the lack of MT in MT-null mice. The results indicate that MT may be one of the essential factors for the induction of carnitine and that the reduction of carnitine induced in MT-null mice must be one of the reasons why MT-null mice tend to be obese (33). The reduction of carnitine with the increase in intensity of restraint in MT-null mice suggests the possibility that, even in wild-type mice, obesity may be induced via reduction in carnitine level under the overlap of loading of stress and lack of MT. Although serum adiponectin levels were significantly decreased in HFD control mice compared with STD control mice and were further decreased in 24-h fasted mice, adiponectin levels in restraint mice were almost the same as those in STD control mice. The decreases in serum adiponectin in C2 and F24 groups as shown in Fig. 10 might be reflects the increase in body and liver weights mainly due to their fat gain (23-25, 34) as shown in Figs. 2 and 4. MT did not affect the induction of serum adiponectin in the present experiment. We observed here two factors, carnitine and adiponectin, concerning lipid metabolism. Further studies are needed for other factors such as triglyceride, cholesterol, and enzymes to clarify the effect of stress on lipid metabolism.

In the present study, we investigated the DNA damage in the cells of four murine organs susceptible to the influence of stress, using mild restraint stress for 15-60 min/day. While some benefits of stress including suppression of body weight gain were observed, mild DNA damages were also detected in the cells of murine organs. Mild and beneficial stress is known to activate brain and promote our health, especially in the elderly (1, 35). It has been suggested that some antioxidative foods (2, 36, 37) may be able to protect the unavoidable mild DNA damage by utilizing the hormetic effect of mild (beneficial) stress (5, 38) as observed in the present study.

MT can be induced by zinc, an essential mineral contained in many foods such as fish and shellfish, meat, nuts and seeds, and so on. Daily intake of zinc from ordinary foods may be effective not only for...
maintaining the physiological functions of the body such as fertility and immunity (39, 40) but also for inducing MT in the body (41, 42). In addition, many antioxidative foods containing many kinds of antioxidant components that protect mild DNA damages induced by moderate stresses should be extensively studied.

**CONFLICT OF INTEREST**

None of the authors have any conflicts of interest to declare.

**ACKNOWLEDGEMENT**

This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan. We wish to thank Drs. T. T. Tsuda and A. Umehara (Tokushima Bunri University, Tokushima, Japan) for their support on carnitine analysis.

**REFERENCES**