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Low-protein diet decreased the adrenal function and spontaneous activity of mice during chronic heat stress

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Abstract

Protein restriction is a well-known risk factor that induces the deterioration of various biological functions. However, little is known about the effects of protein restriction on behavioral markers and the adrenal function of mice exposed to chronic stress. Here we evaluated the effects of a low-protein diet on the spontaneous activity and adrenal function of chronic heat-stressed mice. ICR mice were fed a control diet (20% protein) or a low-protein diet (10% protein) for 14 consecutive days. From the 10th day of the diet period, the mice were repeatedly exposed to a temperature condition of 35 ± 1 °C for 2 hr/day for four consecutive days. The spontaneous activities of the mice were estimated for the behavioral analysis. On the last day, we performed a blood collection test and an ACTH stimulation test for adrenal function analysis. For the blood collection test, mice were exposed to heat stress again for 2 hr, and blood was collected immediately after this heat stress. We measured the plasma levels of corticotropin releasing hormone, adrenocorticotropin (ACTH), and corticosterone. For the ACTH stimulation test, cosyntropin was intraperitoneally administered, and the plasma corticosterone levels were measured. The spontaneous activity of the low-protein mice was significantly lower than that of the control mice during the dark period of heat stress. The plasma corticosterone levels were greatly

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increased by heat stress, with no significant difference between the control and low-protein groups. The ACTH stimulation test revealed that the plasma corticosterone concentration of the heat-stressed low-protein mice was significantly lower than that of the heat-stressed controls. In conclusion, the low-protein diet decreased the spontaneous activity and the adrenal function of mice during heat stress, which implies that protein restriction during chronic heat stress induces fatigue by reducing the adrenal function.

Keywords: Nutrition, Physiology, Endocrinology, Food science, Biochemistry

1. Introduction

Protein malnutrition is a well-known risk factor that induces the deterioration of various biological functions. Protein restriction has been reported to lead to an increase in energy expenditure and the activation of thermogenesis in brown adipose tissue, as evidenced by an increase in catecholamine levels [1]. Another study demonstrated that protein restriction caused a disruption in the crosstalk between the protein kinase A and protein kinase C signaling pathways and, consequently, in the secretory synergism in islets [2]. Protein malnutrition also affects adrenocortical stress response activity [3, 4, 5, 6, 7]. Herbert et al. reported that adrenocorticotropin (ACTH) secretion and adrenocortical physiology were stimulated under conditions of experimentally induced protein malnutrition [3]. Carsia et al. showed that persistent protein malnutrition increases circulating plasma corticosterone concentrations by increasing the relative adrenal weight, adrenocortical cell steroidogenic capacity, and cellular sensitivity to ACTH [4]. Jacobson also demonstrated that protein malnutrition increased pituitary-adrenocortical activity by specifically increasing the drive for ACTH synthesis and secretion [5].

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a key component of the physiological response to stress [8]. Stress initiates a cascade of events, beginning with the stimulation of hypothalamic corticotropin-releasing hormone (CRH) by the central nervous system, which increases pituitary ACTH secretion and finally glucocorticoid (cortisol in humans, corticosterone in rodents) production from the adrenal gland [9]. Cortisol and corticosterone increase blood pressure and blood glucose [10, 11] and relieve inflammation [12], and these reactions are considered to be responses to stressors. However, exposure to chronic stress downregulates adrenal function in animals and humans. Exposure to chronic stress was reported to cause a lower cortisol awakening response, which indicates a hypoactive HPA axis [13]. Adrenal insufficiency leads to fatigue, accompanied by a lack of stamina, loss of energy, and increased irritability [14, 15]. It is thus important to support and maintain adrenal function in the context of chronic stresses.

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There have been many investigations of heat stress, including the physiological responses to heat stress, such as hypoglycemia [16], increases in blood pressure and heart rate [17, 18], and decreases in food consumption and body weight gain [19]. Increased blood glucocorticoid levels due to heat stress treatment have also been reported [20, 21]. It is possible that protein restriction worsens the depression of adrenal function and the fatigue caused by chronic heat stress. However, little is known about the effects of a low-protein diet on behavioral markers and the adrenal function of mice exposed to chronic heat stress. Here we evaluated the spontaneous activity of chronic heat-stressed mice subjected to a diet with 10% protein as a behavioral analysis. We also investigated the effects of the low-protein diet and chronic heat stress on the adrenal function of mice by conducting blood tests after heat stress and by conducting an ACTH stimulation test, which is an established method for assessing adrenal function [22, 23, 24].

2. Materials and methods

2.1. Animal experiments

Six-week-old male ICR mice were obtained from Japan SLC (Shizuoka, Japan) and housed in plastic cages under controlled temperature (22 ± 1 °C), humidity (55 ± 15%), and a 12-hr light/dark cycle for 1 week prior to the commencement of the experiments. All animal experiments were approved by the Meiji Corporation Ethics Committee for Animal Care and Use (Tokyo) and were performed in accordance with the Meiji Corporation Guidelines for Animal Care and Use.

2.2. Measurement of spontaneous activity

The experimental protocol is shown in Fig. 1A. We randomly divided mice into two groups: the control mice were fed a diet with 20% protein, and the low-protein mice were fed a diet with 10% protein for 14 consecutive days. The energy difference due to the reduction of dietary protein was compensated for by carbohydrate (Table 1). The spontaneous activities of the mice during the light and dark periods were estimated for 14 days with an ACTIMO-10 activity monitoring system (Shinfactory, Fukuoka, Japan). The body weight and food and water intake of each mouse were recorded daily. From the 10th day of the diet period, mice were exposed repeatedly to an ambient temperature of 35 ± 1 °C in an incubator (HC-100, Shinfactory) for 2 hr (9:00−11:00) per day for four consecutive days. The mean data from the four days before the heat stress period were used as baseline activity data.

2.3. Blood collection after heat stress

The experimental protocol is shown in Fig. 1B. We randomly divided the mice into four groups: no-heat-stress control, no-heat-stress low-protein, heat-stressed control,

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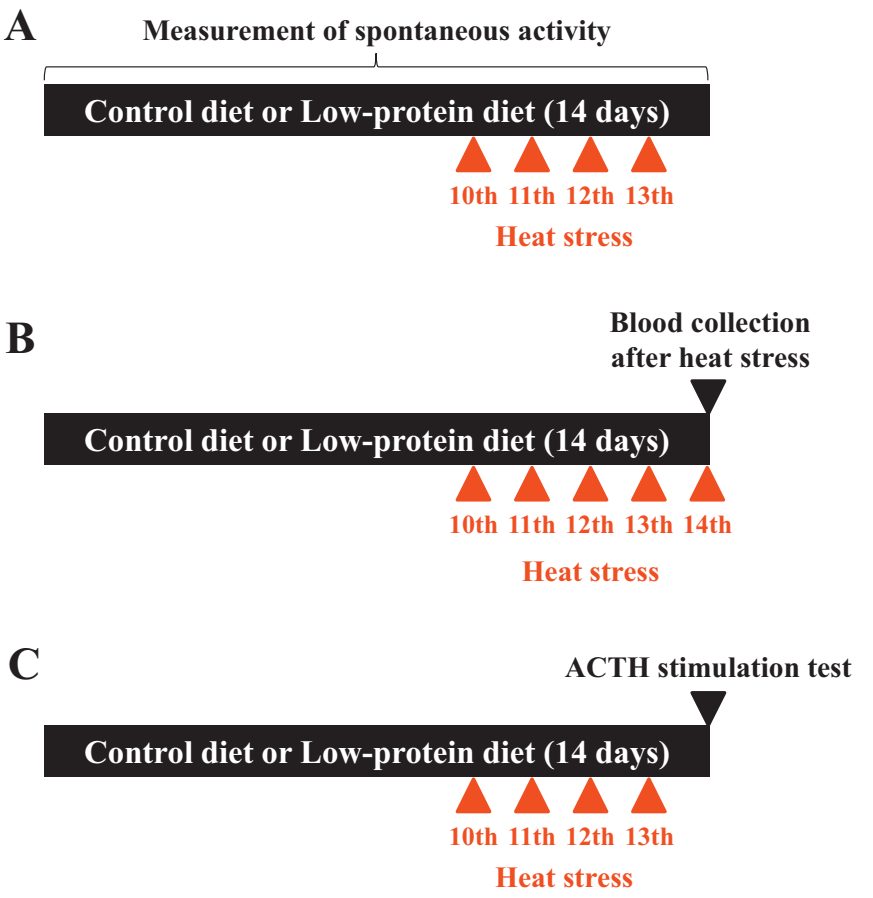

Fig. 1. The experimental protocol. (A) Measurement of spontaneous activity, (B) blood collection after heat stress, (C) ACTH stimulation test.

Table 1. Compositions (%) of the control and low-protein diets.

Ingredient	Control	Low-protein
Casein	20	10
DL-methionine	0.30	0.15
Corn starch	15.00	25.15
Sucrose	50	50
Cellulose	5	5
Corn oil	5	5
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
Choline bitartrate	0.2	0.2

and heat-stressed low-protein. For 14 consecutive days, the control mice were fed a diet with 20% protein, and the low-protein mice were fed a diet with 10% protein. From the 10th day of the diet period, the heat-stressed mice were repeatedly exposed to the above-mentioned heat stress for four consecutive days. On the 14th day, the

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two groups of heat-stressed mice were exposed to heat stress again for 2 hr. Immediately after this heat stress, the mice were anesthetized with isoflurane, and blood was collected by cardiac puncture. The no-heat-stress mice were also anesthetized with isoflurane, and blood was collected by cardiac puncture. Blood samples were centrifuged for 15 min at 12,000 rpm. Plasma samples were collected and frozen at −80 °C.

2.4. ACTH stimulation test

The experimental protocol is shown in Fig. 1C. Mice that were randomly divided into four groups were fed a control diet or a low-protein diet for 14 days and exposed to heat stress for four consecutive days, which is the same protocol that was followed for the *blood collection after heat stress*. On the 14th day, cosyntropin (Daiichi-Sankyo, Tokyo), a synthetic derivative of ACTH, was intraperitoneally administered at a dose of 100 μg/kg body weight. Blood samples were collected from a tail vein 30 and 0 min before and 30, 60, and 90 min after the cosyntropin administration, and the blood was then centrifuged for 5 min at 11,000 rpm. Tail vein plasma samples were collected and frozen at −80 °C.

2.5. Plasma measurement

Caval plasma CRH, ACTH, and corticosterone concentrations were assayed using a Corticotropin Releasing Factor Extraction Free EIA Kit (Phoenix Pharmaceuticals, Burlingame, CA), ACTH Extraction Free EIA Kit (Phoenix Pharmaceuticals), and a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI), respectively. Tail vein plasma corticosterone levels were also assayed using a Corticosterone Enzyme Immunoassay Kit. The intra-assay and inter-assay coefficients of variation were <10 and 15%, respectively. The area under the curve (AUC) of the plasma corticosterone levels during the ACTH stimulation test was calculated.

2.6. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The body weight was analyzed using a two-way repeated measures analysis of variance (ANOVA) with diet (control and low-protein) and time (days 0, 4, 7, 10, 11, 12, 13, and 14). Food and water intake before and during heat stress were analyzed using a two-way repeated measures ANOVA with diet (control and low-protein) and time (before heat stress and during heat stress). The spontaneous activity was analyzed using a two-way repeated measures ANOVA with diet (control and low-protein) and time (baseline, heat1, heat2, heat3, and heat4). Plasma CRH, ACTH, and corticosterone levels were analyzed using a two-way ANOVA with diet (control and low-

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protein) and treatment (no-heat and heat-stressed). The corticosterone levels during the ACTH stimulation test were analyzed using a three-way repeated measures ANOVA with diet (control and low-protein), treatment (no-heat and heat-stressed), and time (-30, 0, 30, 60, and 90 min). Significant interactions were further investigated by simple main effects analyses. A Student's t-test was used for the comparisons of pairs of groups. Differences with p-values <0.05 (*) or $p < 0.01$ (**) were considered significant.

3. Results

3.1. The effects of the low-protein diet on body weight, food intake, and water intake before and during heat stress

The low-protein diet had no effect on the final body weight or the total water intake of the mice during the experiment. The total food intake of the low-protein mice was significantly higher than that of the control mice, whereas the total protein intake of the low-protein mice was markedly lower than that of the control mice (Table 2). A two-way repeated measures ANOVA (diet $\times$ time) was conducted for the body weight of mice and revealed no significant main effect for diet ($p = 0.632$), a significant main effect for time ($p < 0.01$), and no significant interaction between diet and time ($p = 0.483$), which indicates that heat stress inhibited the increase in body weight in both the control and low-protein groups (Fig. 2). A two-way repeated measures ANOVA (diet $\times$ time) was conducted for food intake and revealed a significant main effect for diet ($p < 0.01$) and time ($p < 0.01$) and no significant interaction between diet and time ($p = 0.486$), which indicates that heat stress decreased the food intake of both the control mice and the low-protein mice (Table 3). A two-way repeated measures ANOVA (diet $\times$ time) for the water intake revealed no significant main effects or interaction.

Table 2. The effects of the low-protein diet on the body weight, food intake, and water intake of mice.

Variable	Control	Low-protein
Initial body weight, g	30.4 ± 0.3	30.5 ± 0.2
Final body weight, g	36.8 ± 0.8	37.6 ± 0.5
Total food intake, g	71.8 ± 2.7	82.6 ± 2.0**
Total food intake, g/g body weight	1.95 ± 0.07	2.20 ± 0.04**
Total water intake, mL	105 ± 16	105 ± 13
Total water intake, mL/g body weight	2.85 ± 0.38	2.79 ± 0.33
Protein intake, g	14.4 ± 0.54	8.26 ± 0.20**
Protein intake, g/g body weight	0.390 ± 0.014	0.220 ± 0.004**

Values are expressed as the mean ± SEM ($n = 8$). **$p < 0.01$ compared to the control group.

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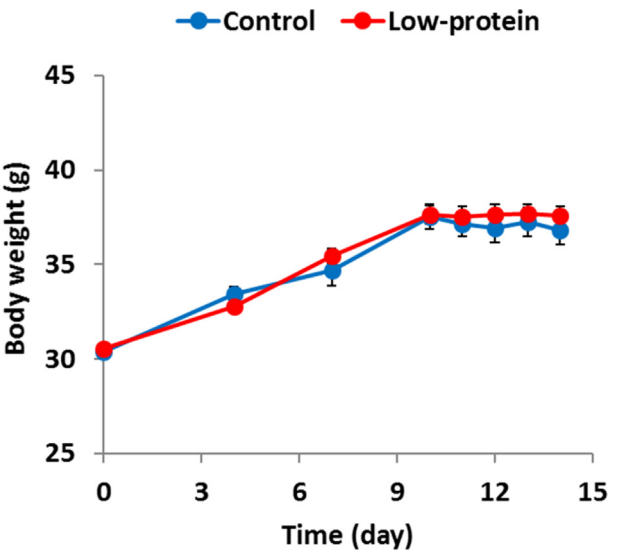

Fig. 2. The effects of the low-protein diet on body weight before and after heat stress. Values are expressed as the mean ± SEM (n = 8).

Table 3. The effects of the low-protein diet on food and water intake before and during heat stress.

Variable	Control	Low-protein
Food intake before heat stress, g/day	5.63 ± 0.17	6.38 ± 0.17
Food intake during heat stress, g/day	4.32 ± 0.14	4.93 ± 0.11
Water intake before heat stress, mL/day	7.85 ± 1.56	8.39 ± 1.25
Water intake during heat stress, mL/day	6.92 ± 0.66	5.72 ± 0.58

Values are expressed as the mean ± SEM (n = 8). "Before heat stress" and "during heat stress" means "from the 1st day to the 9th day" and "from the 10th day to the 14th day".

3.2. The effects of the low-protein diet on spontaneous activity after heat stress

A two-way repeated measures ANOVA (diet × time) for spontaneous activity during the light period revealed no significant main effects or interaction (Fig. 3A). A two-way repeated measures ANOVA (diet × time) was conducted for spontaneous activity during the dark period and revealed a significant interaction between diet and time ($p < 0.05$). Simple main effect analyses revealed that the spontaneous activity of the low-protein mice was significantly lower than that of the control mice during the dark period of the 1st and 4th days of heat stress (Fig. 3B).

3.3. The effects of the low-protein diet on plasma CRH, ACTH, and corticosterone levels after heat stress

A two-way repeated measures ANOVA (diet × treatment) was conducted for the plasma CRH level and revealed no significant main effect for diet ($p = 0.597$), a

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significant main effect for time ($p < 0.01$), and no significant interaction between diet and treatment ($p = 0.165$), which indicates that the plasma CRH levels were increased by heat stress. A two-way repeated measures ANOVA (diet × treatment) was conducted for the plasma ACTH level and revealed no significant main effect for diet ($p = 0.062$), a significant main effect for time ($p < 0.01$), and no significant interaction between diet and treatment ($p = 0.076$), which indicates that the plasma ACTH levels were decreased by heat stress. A two-way repeated measures ANOVA (diet × treatment) was conducted for the plasma corticosterone level and revealed no significant main effect for diet ($p = 0.767$), a significant main effect for time ($p <$

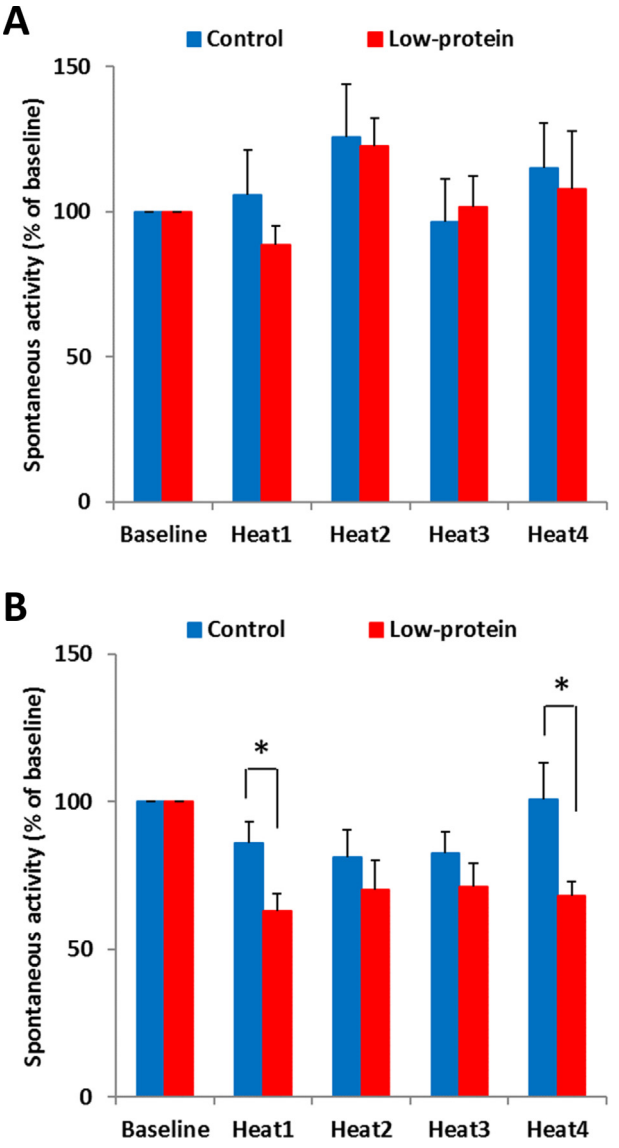

Fig. 3. The effects of the low-protein diet on spontaneous activity after heat stress. The spontaneous activities of the mice during (A) the light period and (B) the dark period. The mean values of the last four days before the heat stress period were used as the baseline activity. Values are expressed as the mean ± SEM ($n = 8$). *$p < 0.05$ vs. the control group.

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0.01), and no significant interaction between diet and treatment ($p = 0.989$), which indicates that the plasma corticosterone levels were increased by heat stress. These analyses also revealed that there were no significant differences in the plasma CRH, ACTH, or corticosterone levels between the control mice and the low-protein mice (Table 4).

3.4. The effects of the low-protein diet on adrenal function after heat stress

A three-way repeated measures ANOVA (diet × treatment × time) was conducted for the plasma corticosterone level during the ACTH stimulation test and revealed a significant interaction between diet and treatment ($p < 0.05$). Simple main effect analyses revealed that no significant difference in the plasma corticosterone levels during the ACTH stimulation test was observed between the no-heat-stress controls and the heat-stressed controls, whereas the plasma corticosterone concentrations of the heat-stressed low-protein mice were significantly lower than those of the no-heat-stress low-protein mice (Fig. 4A). In addition, no significant difference in the plasma corticosterone levels during the ACTH stimulation test was observed between the no-heat-stress controls and the heat-stressed low-protein mice, whereas the plasma corticosterone concentrations of the heat-stressed low-protein mice were significantly lower than those of the heat-stressed control mice (Fig. 4A). The AUC of the plasma corticosterone concentration of the heat-stressed low-protein mice was significantly lower than that of the no-heat-stress low-protein mice and that of the heat-stressed controls (Fig. 4B).

4. Discussion

Our investigation of the effects of a low-protein diet and heat stress on the behavioral and physiological actions of mice revealed the following. The body weights of both the controls and low-protein mice were higher during the no-heat-stress period, and during the heat-stress period, the increases in the body weights of the controls and low-protein mice were potently suppressed. Thus, whether the mice were fed the control diet or the low-protein diet, the body weight increase was suppressed during

Table 4. Effects of the low-protein diet on the plasma CRH, ACTH, and corticosterone levels after heat stress.

Variable	No-heat Control	No-heat Low-protein	Heat-stressed Control	Heat-stressed Low-protein
Plasma CRH	0.463 ± 0.017	0.534 ± 0.048	0.633 ± 0.044	0.600 ± 0.027
Plasma ACTH	1.53 ± 0.20	2.33 ± 0.31	1.14 ± 0.08	1.17 ± 0.14
Plasma corticosterone	130 ± 16	122 ± 14	401 ± 44	393 ± 29

Values are all ng/mL and are expressed as the mean ± SEM (n = 7–8).

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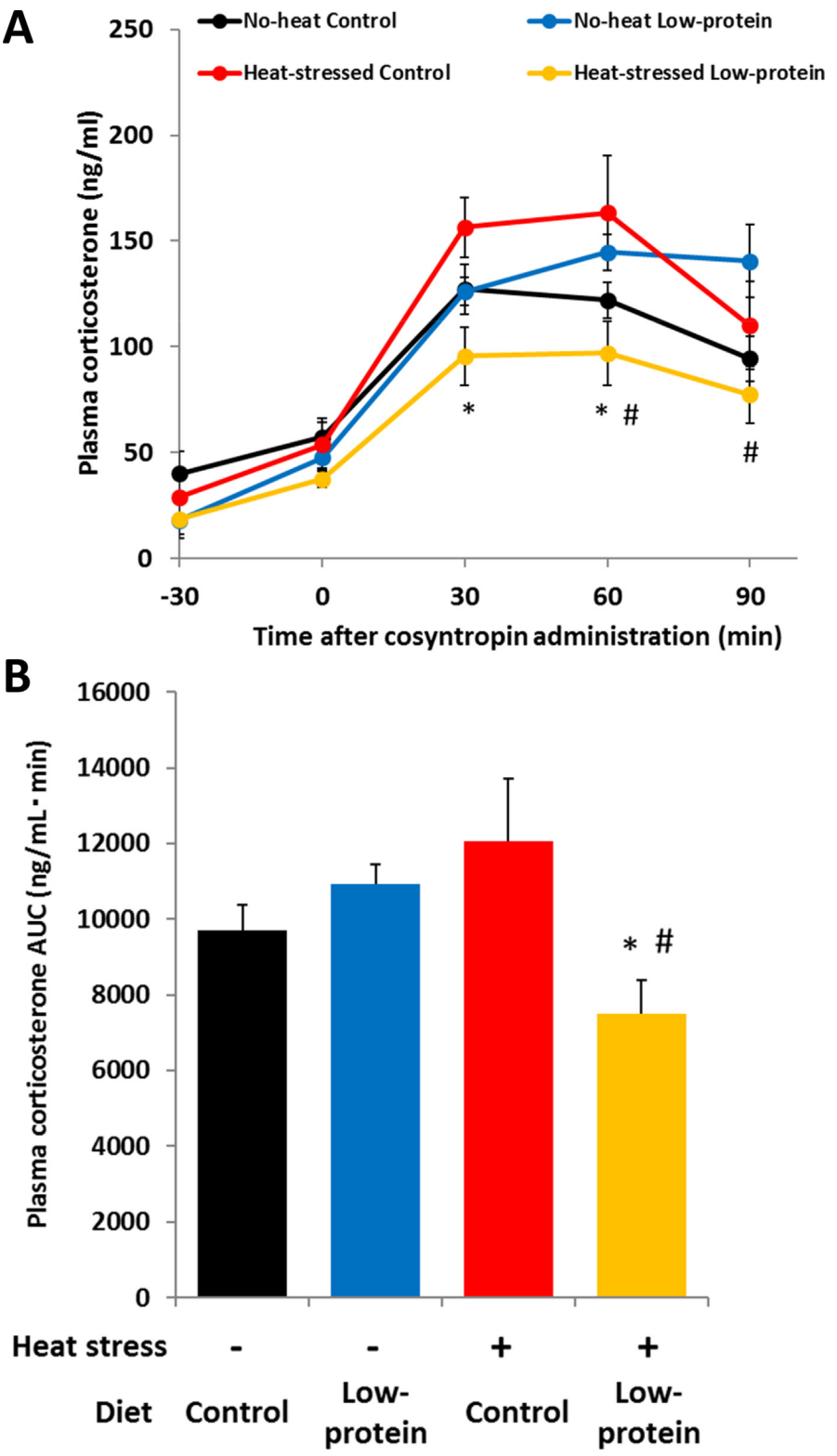

Fig. 4. The effects of the low-protein diet on adrenal function after heat stress. (A) Tail vein plasma corticosterone levels. (B) The AUCs of the plasma corticosterone levels. Values are expressed as the mean ± SEM (n = 5–8). *p < 0.05 vs. the control group. #p < 0.05 vs. the no-heat-stress group.

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heat stress. The food intake of both the control mice and the low-protein mice also decreased during the heat-stress period compared to the no-heat-stress period, which indicates that the suppression of the body weight increase was caused by the decrease in food intake.

Harikai et al. reported that heat treatment resulted in decreased food consumption and body weight gain [20], which is in agreement with our present findings. Several studies have demonstrated that a low-protein diet caused a decrease in body weight [1, 2, 25], but in the present study, there was no significant difference in body weight between the control and low-protein groups. In the above-cited studies, an approximately 6% protein diet was used as the low-protein diet, and the lower protein content could be responsible for the more significant effect of protein malnutrition compared to that observed in the present study, in which a 10% protein diet was used as the low-protein diet. Our use of a 10% protein diet could be the reason the low-protein diet did not induce a decrease in body weight. The food intake of the mice fed the low-protein diet was significantly higher than that of the control mice, which is in accordance with the results of previous studies [1, 2, 25]. On the other hand, the protein intake of the low-protein mice in our experiment was significantly lower than that of the control mice, which indicates that protein restriction was clearly achieved in this study.

Spontaneous activity is generally used for behavioral analyses in animal experiments [26, 27, 28]. Inoue et al. reported that a depression of spontaneous activity is caused by the decline in the motivation to move due to an arising sensation of fatigue [27]. Here we evaluated the effect of a low-protein diet on the spontaneous activity of mice after chronic heat stress (as an index of fatigue). No significant difference in the spontaneous activity of the mice during the light periods occurred due to protein restriction or chronic heat stress. This is likely because rodents sleep mainly during the light period; thus, their spontaneous activity was low during the light period. In the dark period, the spontaneous activity of the low-protein mice was significantly lower than that of the control mice on the 1st and 4th days of heat stress, which indicated that the low-protein diet induced fatigue during chronic heat stress. In addition, there was no significant difference between the baseline activity of the control mice and that of the low-protein mice before heat stress in this study. This suggests that the decreased spontaneous activity of the low-protein mice during heat stress could be associated with the response to heat stress rather than the effect of a low-protein diet-related decrease in muscle function on the ability to move spontaneously.

Exposure to chronic stress has been reported to cause a lower cortisol awakening response and to reflect a hypoactive HPA axis [13]. Adrenal insufficiency leads to fatigue, accompanied by a lack of stamina, loss of energy, and increased irritability [14]. We evaluated whether the above-described fatigue during chronic heat stress accompanied by protein restriction was involved in the depression of adrenal

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function by conducting blood tests and an ACTH stimulation test after heat stress. The post-heat stress blood test revealed that the plasma corticosterone level was dramatically increased by heat stress, indicating that the heat stress in the present study (35 ± 1 °C for 2 hr) was a definite stressor for the mice. However, no significant difference in plasma corticosterone levels were observed between the heat-stressed control mice and the heat-stressed low-protein mice. We suspected that this lack of a significant difference occurred because the increase in corticosterone secretion as a result of the heat stress was too high to evaluate the effect of protein restriction on heat stress-induced corticosterone secretion. A significant difference in plasma corticosterone may be observed between the low-protein group and the control group after being subjected to more moderate stress rather than the heat stress that was used in this study, which should be investigated in future studies.

In contrast, we observed that the plasma ACTH level was low in the condition of heat stress, and there was no significant difference in the plasma ACTH levels between the control mice and the low-protein mice; this might be due to corticosteroid feedback, which is known to decrease the release of ACTH [29]. A significant difference in CRH levels was also not observed between the control and low-protein groups in the condition of heat stress. These results indicate that the heat stress in the present study was sufficient to elevate the plasma corticosterone level in mice, but it might be difficult to evaluate the effects of protein restriction on adrenal function during chronic heat stress by performing blood tests after heat stress.

An ACTH stimulation test is generally used as a method for assessing adrenal function [22, 23, 24]. The ACTH stimulation test of the present study demonstrated that the plasma corticosterone level of the heat-stressed low-protein mice was significantly lower than that of the heat-stressed control mice. The AUC of the plasma corticosterone levels of the heat-stressed low-protein mice was significantly lower than that of the heat-stressed control mice. These results indicate that the low-protein diet worsened the adrenal function of the mice compared to that of the mice with an appropriate amount of protein in their diet during chronic heat stress. Jacobson et al. demonstrated that protein malnutrition increased pituitary-adrenocortical activity by increasing ACTH synthesis and secretion [5]. It was also reported that blood glucocorticoid levels were dramatically increased by heat stress treatment [20, 21]. With the multiple stresses of protein restriction and heat, the adrenal function of mice could deteriorate. Moreover, we observed a correlation between the spontaneous activity during the dark period and the AUC of the plasma corticosterone levels in the ACTH stimulation test, which supports the idea that the deterioration of adrenal function caused a decrease in the spontaneous activity of the mice.

In conclusion, our experimental findings demonstrated that a low-protein diet decreased the spontaneous activity and adrenal function of mice during heat stress,

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which implies that protein malnutrition during chronic heat stress induces fatigue by impairing adrenal function.

Declarations

Author contribution statement

Yuichi Tsuda: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kaori Iwasawa: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Makoto Yamaguchi: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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