Elevation of body temperature is an essential factor for exercise-increased extracellular heat shock protein 72 level in rat plasma

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Ogura Y, Naito H, Akin S, Ichinoseki-Sekine N, Kurosaka M, Kagiki R, Sugura T, Powers SK, Katamoto S, Demirel HA. Elevation of body temperature is an essential factor for exercise-increased extracellular heat shock protein 72 level in rat plasma. Am J Physiol Regul Integr Comp Physiol 294: R1600–R1607, 2008. First published March 26, 2008; doi:10.1152/ajpregu.00581.2007.—This study examined whether the exercise-increased extracellular heat shock protein 72 (eHsp72) levels in rats was associated with body temperature elevation during exercise. In all, 26 female Sprague-Dawley rats (3 mo old) were assigned randomly to control (CON; n = 8), exercise under warm temperature (WEx; n = 9), or exercise under cold temperature (CEx; n = 9). The WEx and CEx were trained at 25°C or 4°C, respectively, for nine days using a treadmill. Before and immediately after the final exercise bout, the colonic temperatures were measured as an index of body temperature. The animals were subsequently anesthetized, and blood samples were collected and centrifuged. Plasma samples were obtained to assess their eHsp72 levels. Only the colonic temperature in WEx was increased significantly (P < 0.05) by exercise. The eHsp72 level in WEx was significantly higher (P < 0.05) than that of either the CON or CEx. However, no significant difference was found between CON and CEx. Regression analyses revealed that the eHsp72 level increased as a function of the body temperature. In another experiment, the eHsp72 level of animals with body temperature that was passively elevated through similar kinetics to those of the exercise was studied. Results of this experiment showed that mere body temperature elevation was insufficient to induce eHsp72 responses. Collectively, our results suggest that body temperature elevation during exercise is important for induction of exercise-increased eHsp72. In addition, the possible role of body temperature elevation is displayed when the exercise stressor is combined with it.

running exercise; warm; cold; ELISA; stress proteins

HEAT SHOCK PROTEINS (HSPS) play important physiological roles in various cells as a molecular chaperone (18). In fact, Hsps are separable into several groups based upon their molecular masses. Of particular interest is the inducible form of the 70-kDa family of Hsps (Hsp72) because Hsp72 can be induced dramatically by stress. It is therefore considered to be the major inducible form of Hsps in living organisms (15).

Many stressors can increase the Hsp72 level in cells: one is physical exercise. Physical exercise imposes stress in many ways, as thermal, mechanical, metabolic, or oxidative stress, on the cells of various organs. Indeed, the Hsp72 level increases after both acute and chronic physical exercise in various organs of rodents (2, 3, 8, 10, 21, 24, 27) and humans (6, 12, 16, 17, 23). The mechanisms of induction of Hsp72 by exercise in the cell have been largely understood. Exercise-related stressors stimulate trimerization of the heat shock factor; the heat shock factor trimer binds a heat shock element in the nucleus and upregulates the Hsp72 gene expression and protein synthesis (1, 15). This increased Hsp72 is considered to be a helper for the exercise adaptation and maintenance of cellular homeostasis via the chaperone function (15, 26).

On the other hand, recent studies have shown that the exercise stress also increases Hsp72 in the circulation (14, 20, 34, 35). For example, Walsh et al. (35) showed that 60 min of running exercise (60% VO2 peak) increased serum Hsp72 level in human subjects. In addition, Suzuki et al. (34) reported that the plasma Hsp72 level was elevated 22-fold after the Ironman triathlon race. This Hsp72 in the blood is known as an extracellular Hsp72 (eHsp72); it is suggested that eHsp72 works as a signal on an immune system (1). Compared with cellular Hsp72 regulation, however, the mechanisms and origin of the exercise-increased eHsp72 remain controversial and have yet to be explored, although results of some studies suggest that the increase of eHsp72 during exercise is provided by their release from some organs (4, 5, 14).

In general, the primary factor to stimulate Hsp72 production is an elevation of the cell temperature (19, 30). Indeed, the production of Hsp72 was accelerated in some organs when exercise and training were combined with the elevation of body temperature (9, 33). In this regard, recent data might imply that eHsp72 is also influenced by elevated temperature. For instance, one study showed that plasma eHsp72 levels in runners with exertional heat illness were higher than those of normal runners at the end of a 14-km running event (31). Another study has reported that heat stress (43°C) on cultured cells or human peripheral blood mononuclear cells (PBMCs) increased the Hsp72 content in both the intracellular fraction and cultured cell medium (13). Consequently, these studies suggest that increased eHsp72 during exercise is associated with the elevation of body temperature during exercise. On the basis of the reasoning described above, this study was intended to examine whether the exercise-increased eHsp72...
level is associated with the elevation of body temperature during exercise.

MATERIALS AND METHODS

Experimental Design

This study was composed of two separate experiments: experiment 1 was the main experiment to investigate the effect of elevation of body temperature on the eHsp72 response induced by exercise, and experiment 2 was undertaken to examine the effect of elevation of body temperature with similar kinetics to the exercise on eHsp72 for supporting experiment 1.

All procedures of this experiment were approved by the Juntendo University Animal Care and Use Committee and conducted according to American Physiological Society guiding principles in the care and use of animals and guiding principles for the care and use of animals in the field of physiological sciences by Physiological Society of Japan. All animals used in this study were obtained from a licensed laboratory animal vendor (Japan SLC, Shizuoka, Japan). On arrival in our laboratory, all animals were fed standard rat chow and water ad libitum and housed on a 12:12-h light-dark photoperiod (lights off 0900-2100) in an environment-controlled room (23 ± 1°C, 55 ± 5% relative humidity). In both experiments, all exercise or heating interventions were undertaken at the same daily time with counter-balanced order to eliminate the effect of a circadian rhythm.

Experiment 1

Animals. Twenty-six female Sprague-Dawley rats (3 mo old) were used in experiment 1. After arrival to our animal facility, the animals were familiarized with treadmill running and then randomly assigned to one of following groups: control (CON, n = 8, 219.2 ± 6.1 g), exercise under warm temperature (WEx, n = 9, 221.7 ± 8.5 g), and exercise under cold temperature (CEX, n = 9, 219.4 ± 5.0 g) groups.

Training protocol. To examine the influences of body temperature elevation by exercise, we performed the previously established training protocol by Hamilton et al. (9). They showed that the cold condition (4°C) could block elevation of the body temperature during exercise and prevent exercise-induced Hsps in cardiac muscle. Therefore, this training model was considered adequate to investigate the effects of elevation of body temperature on Hsps induction in exercise-elevated body temperature (37). Immediately after the final bout of training (the time was ∼1230–1330), the colonic temperature of the WEx and CEx animals was measured (after exercise). Then the animals were anesthetized using pentobarbital sodium (50 mg/kg). After reaching a surgical plane of anesthesia, blood samples were collected with EDTA from an abdominal vein as quickly as possible. After taking the blood samples, the liver and gastrocnemius muscle were removed quickly, weighed, then frozen with liquid nitrogen, and stored at −85°C until analysis of the Hsp72 and heat shock protein cognate 73 (Hsc73) contents. Simultaneously, CON animals were killed without a second measurement of their colonic temperature.

Experiment 2

Animals. Experiment 2 was designed to examine the effect of elevation of body temperature with similar kinetics to the exercise on eHsp72 for separating the influences of the influences of “exercise” and “temperature elevation.” Twenty-one female Sprague-Dawley rats (3 mo old) were used in experiment 2. After an arrival to our animal facility, the animals were familiarized with treadmill running and then randomly assigned to one of following groups; control (CON-II, n = 7, 226.3 ± 7.7 g), exercise (EXE, n = 7, 219.8 ± 4.8 g), and passive heating (HEAT, n = 7, 218.9 ± 4.0 g) groups. During the following interventions, CON-II animals were placed beside the animal treadmill and their colonic temperature was measured simultaneously with the other two groups.

Exercise protocol. EXE animals were exercised on an animal treadmill for 60 min. The exercise protocol is shown in Table 2. This exercise was conducted during the active period of the rat in a dark room. Colonic temperatures were measured before the exercise (basal), at 30 min of exercise, and immediately after the end of exercise (after exercise). The blood samples were taken as soon as possible after the exercise. The liver and gastrocnemius muscle were also removed.

Passive heating protocol. HEAT animals were passively heated in a heat chamber for 60 min. The animal colonic temperatures were increased to mimic the kinetics of elevation of body temperature in EXE animals, by controlling the chamber temperature. Colonic temperatures were measured at the same time point of EXE animals. Immediately after the passive heating, blood samples were taken. Then, the liver and gastrocnemius muscle were removed.

Measurements

Blood handling. The blood samples were separated into two parts. One part was centrifuged to obtain the plasma at 4,000 rpm for 10 min. These plasma samples were stored at −85°C until eHsp72 analysis. Another part was used for measurement of hemoglobin concentration and hematocrit as an index of a dehydration status of animals using an automated blood cell counter (MEK-6318; Nihon Kohden, Tokyo, Japan). Blood samples of control animals in each experiment were taken without exercise or passive heating interventions.

eHsp72 determination in plasma. The eHsp72 levels in the plasma were measured using a commercially available enzyme-linked immunoassay kit (EKS-700; Stressgen Biotechnologies, BC, Canada), according to the manufacturer’s instructions. In brief, 100 µL of diluted plasma samples were applied to the wells of Hsp72 immunoassay plate bound with mouse monoclonal antibody specific to Hsp72 and incubated for 2 h. After repeated washing, the captured Hsp72 was incubated with rabbit polyclonal Hsp72-specific biotin-conjugated antibody for 1 h. After repeated washing, the plate was incu-
bated with an avidin-horseradish peroxidase conjugate for 1 h. After repeated washing, the plate was developed with tetramethylbenzidine substrate for 10 min and stopped with an acid stop solution. The absorbance of each well was then measured using a microplate reader (Multiscan MS; Thermo Labsystems, Helsinki, Finland) at 450 nm.

The same amount of protein was loaded on each well of SDS-PAGE gel (10% for separation and 4% for stack) and then run at a constant voltage of 100 V for 2 h. After the separation, the proteins were transferred to nitrocellulose membranes (pore size 0.45 μm; Bio-Rad) at a constant voltage of 100 V for 1 h. After the transfer, membranes were blocked for 1 h using 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (T-TBS) and then incubated with alkaline phosphatase-conjugated monoclonal antibodies that were specific to Hsp72 or Hsc73 (SPA-810AP, SPA-815AP, diluted 1:3,000; Stressgen), in T-TBS with 2.5% nonfat dry milk overnight at 4°C. The membranes were subsequently washed using T-TBS and TBS; they were then reacted with bromochloroindolyl phosphate-nitro blue tetrazolium substrate (Bio-Rad) at room temperature. Quantification of each band was performed using computerized densitometry (ImageJ, ver. 10.2; National Institutes of Health, Bethesda, MD).

Table 2. Exercise protocol in experiment 2

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Animals were exercised using a motor-driven animal treadmill for a duration of 60 min with each experimental condition under 25°C condition.

RESULTS

Experiment 1

No significant difference was found in body weights after the training period among groups (CON, 228.4 ± 6.7 g; WEx, 240.1 ± 15.3 g; CEx, 234.3 ± 11.0 g). No significant difference was found in hemoglobin concentrations among groups (CON, 13.2 ± 0.5 g/dl; WEx, 12.8 ± 0.4 g/dl; CEx, 12.7 ± 0.8 g/dl). No significant difference was found in hematocrit among groups (CON, 37.1 ± 1.6%; WEx, 36.1 ± 1.0%; CEx, 35.2 ± 2.2%).

Figure 1 indicates the changes in colonic temperature before and after exercise in WEx and CEx animals. Two-way ANOVA revealed significant (F1,32 = 41.34, P < 0.0001) interaction between time and temperature conditions. The colonic temperature after the exercise in WEx animals was significantly (P < 0.001) higher than their basal level. The colonic temperature after the exercise in WEx animals was also significantly (P < 0.001) higher than that of CEx animals. On the other hand, the colonic temperature after the exercise in CEx animals was not different from their basal level. The basal colonic temperature of CON animals was 38.0 ± 0.3°C.

Figure 2 shows the results of eHsp72 concentration. ANOVA revealed significant F ratio (F2,23 = 11.57, P < 0.0004), and the WEx animals showed significantly higher eHsp72 level than those of CON (P < 0.001) and CEx (P < 0.01) animals. However, significant differences were not found between CON and CEx animals.

The Hsp72 levels in the liver and red and white gastrocnemius muscle are expressed as a percentage to each CON value; they are shown in Fig. 3. ANOVA revealed significant F ratios in the liver (F2,23 = 30.54, P < 0.0001), red (F2,23 = 15.07, P < 0.0001), and white (F2,23 = 33.15, P < 0.0001) gastrocnemius muscles. In the Hsp72 level of the liver (Fig. 3A), WEx animals showed a significantly higher Hsp72 level than those of CON (P < 0.001) and CEx (P < 0.001) animals. Although Hsp72 of the liver in CEx animals tended to be higher than in CON animals, no statistically significant difference was found between CON and CEx animals. Regarding the red gastroc-
muscle (Figs. 3B), WEx animals showed significantly higher levels than CON ($P < 0.001$) and CEx ($P < 0.01$) animals. For the white gastrocnemius muscle (Figs. 3C), WEx animals showed significantly higher levels than CON ($P < 0.001$) and CEx ($P < 0.001$) animals. Similarly to the liver, although the Hsp72 of red and white gastrocnemius muscles in CEx animals tended to be higher than CON animals, no statistically significant difference was found between CON and CEx animals. No statistically significant difference was found among groups in Hsc73 levels of liver and red and white gastrocnemius muscles (Table 3 and Fig. 4).

Figure 5 shows the relationship of colonic temperatures to eHsp72 levels in exercising animals. In this analysis, colonic temperatures from WEx and CEx animals are from temperatures taken immediately after exercise. The quadratic regression analysis indicated a significant relationship between colonic temperatures and eHsp72 levels. ($y = 0.233x^2 - 17.939x + 346.1, r^2 = 0.5772, F_{2,15} = 10.24, P < 0.02$).

**Experiment 2**

No significant difference was found in hemoglobin concentrations (CON-II, 13.9 ± 0.6 g/dl; EXE, 14.0 ± 0.9 g/dl; HEAT, 14.0 ± 0.9 g/dl) and hematocrit (CON-II, 37.2 ± 1.7%; EXE, 36.1 ± 1.9%; HEAT, 36.9 ± 2.4%) among groups.

Figure 6 portrays the changes in colonic temperature at time points of before, at 30 min, and after exercise in three groups. Two-way ANOVA revealed significant ($F_{4,36} = 11.54, P < 0.0001$) interaction between time and group conditions. The colonic temperatures at the time point of 30 min and after exercise in both EXE and HEAT animals were significantly ($P < 0.001$) higher than the respective basal values. The colonic temperatures at the time point of 30 min and after exercise in both EXE and HEAT animals were also significantly ($P < 0.001$) higher than those of CON-II animals. However, no significant differences were found between the colonic temperatures of EXE and HEAT animals at the any time point. The colonic temperature of CON-II animals did not change at any time point compared with basal values.

Figure 7 shows the eHsp72 concentration of CON-II, EXE, and HEAT animals. ANOVA revealed significant $F$ ratio ($F_{2,18} = 8.85, P < 0.001$), and the EXE animals showed significantly higher eHsp72 level than those of CON-II ($P < 0.01$) and HEAT ($P < 0.05$) animals. No statistically significant difference was found between CON-II and HEAT animals. Table 4 shows the Hsp72 levels in the liver and red and white gastrocnemius muscles. No statistically significant difference was found among groups in Hsp72 levels of liver and red and white gastrocnemius muscles. In Hsc73 levels, no statistically significant difference was found among groups in liver and red and white gastrocnemius muscles.

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**Fig. 2.** Extracellular heat shock protein 72 (eHsp72) concentrations of animals in control (CON, $n = 8$), and exercise under 25°C (WEx, $n = 9$) and 4°C (CEx, $n = 9$) conditions. Values are expressed as means (SD). ***$P < 0.001$ vs. CON, §§§$P < 0.01$ vs. CEx.

**Fig. 3.** Heat shock protein 72 (Hsp72) levels of control (CON, $n = 8$), and exercise under 25°C (WEx, $n = 9$) and 4°C (CEx, $n = 9$) conditions. A, B, and C: denote liver and red and white gastrocnemius muscles, respectively. Values are expressed as means (SD). **$P < 0.01$ vs. CON; ***$P < 0.001$ vs. CON; §§§$P < 0.01$ vs. CEx; §§§§$P < 0.001$ vs. CEx.
**Principle Findings**

This study examined whether an extracellular heat shock protein 72 (eHsp72) level is associated with the elevation of body temperature during exercise. The eHsp72 level in animals trained under WEx was significantly increased by exercise. However, such exercise-increased eHsp72 level was completely inhibited in animals trained under CEx. In addition, this eHsp72 level increased as a function of the body temperature. Moreover, animals with passive heating, which elevates body temperature having a similar kinetics to the exercise, produced no significant increase of the eHsp72. These results suggest that the elevation of body temperature during exercise is an essential factor of exercise-induced eHsp72 responses. Additionally, these results suggest that the possible role of the body temperature elevation is displayed when the exercise stressor is combined with it.

**Possible Mechanisms to Increase the eHsp72**

The precise mechanisms for increased eHsp72 in circulation have not been identified. Nevertheless, it is reasonable to presume that eHsp72 is released from the stressed cells of any organ because the production of this protein requires transcriptional and translational steps in the cells (1, 15). Indeed, some studies using human subjects have shown the release of eHsp72 from organs during exercise. Febbraio et al. (4, 5) demonstrated that the liver releases eHsp72 by calculating the hepatosplanchnic venous-arterial difference of eHsp72 level during a single bout of cycling exercise. In addition, Lancaster et al. (14) reported that eHsp72 is released from the brain during knee extension exercise by measuring jugular venous-arterial differences of the eHsp72 level. These studies imply that, at least in part, the exercise-increased eHsp72 originates from some organs of exercised animals.

In the present study, eHsp72 level of WEx animals was higher than control (CON) animals after exercise, but such was not the case in CEx animals. These facts indicate that elevation of the body temperature is necessary to induce the exercise-increased eHsp72 response. Although this study did not identify the exact mechanisms responsible for the increased eHsp72 by the elevation of body temperature, a possibility is “receptor-mediated” regulation of eHsp72. Johnson et al. (11) reported that tail shock stressor exposure stimulated an increase in eHsp72 in the blood and that administration of an /H92511-adreno-receptor antagonist blocks the increase. Johnson et al. (11) also

**DISCUSSION**

This study examined whether an extracellular heat shock protein 72 (eHsp72) level is associated with the elevation of body temperature during exercise. The eHsp72 level in animals trained under WEx was significantly increased by exercise. However, such exercise-increased eHsp72 level was completely inhibited in animals trained under CEx. In addition, this eHsp72 level increased as a function of the body temperature. Moreover, animals with passive heating, which elevates body temperature having a similar kinetics to the exercise, produced no significant increase of the eHsp72. These results suggest that the elevation of body temperature during exercise is an essential factor of exercise-induced eHsp72 responses. Additionally, these results suggest that the possible role of the body temperature elevation is displayed when the exercise stressor is combined with it.

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![Fig. 4. Representative immunoblotting of heat shock protein cognate 73 of liver and red and white gastrocnemius muscles. CON, WEx, and CEx mean control animals and those exercised under 25°C and 4°C conditions, respectively.](image-url)
showed that α1 and not β1 and β2 adrenoreceptor blocker (prazosin, 2.0 mg/kg) inhibited the stress-induced increase of eHsp72 level and that α1 agonist administration in the absence of tail shock stress increased eHsp72 level. Furthermore, they (11) showed that the elevation of eHsp72 with tail-shock stress was strengthened by a high level of available norepinephrine in circulation, which acts via α1-adrenoreceptor. These results indicate that the norepinephrine-α1-adrenoreceptor system is important for regulating the circulating eHsp72 level. According to previous studies, the intensity of our exercise is above the rats’ lactate threshold (28); this intensity of exercise increases the circulating norepinephrine level (32). However, eHsp72 in CEx animals implies that exercise itself did not increase the eHsp72 level. In this regard, it has been shown that body temperature influences the circulating norepinephrine level during exercise. Indeed, results of previous studies suggest that the plasma norepinephrine was augmented by prolonged exercise combined with heat treatment compared with thermoneutral exercise condition (29). In our study, the body temperature in WEx animals reached ≥40°C, but that of CEx animals remained at a basal level after the exercise (Fig. 1). These facts suggest that the circulating norepinephrine level was expected to be greater in WEx animals than in CEx animals, although we were unable to measure the norepinephrine level of animals. As described above, because norepinephrine stimulates the increase in eHsp72 via α1-adrenoreceptor (11), WEx animals might show higher eHsp72 levels by exercise than either CON animals or CEx animals. Further pharmacological experiments using blockade of α1-adrenoreceptor are necessary to provide detailed information related to the influences of “receptor-mediated” process on exercise-increased eHsp72.

Another potent mechanism of eHsp72 increasing in circulation is a transportation of this protein on the cell plasma membrane via an exosome. Lancaster and Febbraio (13) showed that the elevation of cell temperature (≥40°C) increased eHsp72 in human PBMCs directly in an exosome-dependent manner, irrespective of the intracellular Hsp72 content. They (13) also found that these eHsp72 releases were temperature dependent. In this study, the colonic temperature in WEx animals became greater than 40°C during the exercise. Therefore, this exosomal pathway might be related to our results.

Results of this study showed that an increase in eHsp72 was correlated significantly to increased body temperature during exercise (Fig. 5). That fact suggests the existence of a certain level of body temperature that dramatically increases the eHsp72 level. In various tissues, the threshold level of temperature is known to increase the Hsp72 level. For example, Ruell et al. (30) showed that at least 40°C (or more than 3°C from basal level) of body temperature was necessary to increase the Hsp72 level in the cardiac muscle, liver, or gut tissue. They also found that a heat stress of less than 39°C was incapable of inducing the Hsp72 synthesis in those tissues, even when using 1 h of heating (30). Similar to Ruell et al. (30), our quadratic regression curve indicates that eHsp72 level is rapidly increased from about 39.5–40°C of colonic temperature (Fig. 5). More research in this area is clearly needed; however, our result seems to suggest a threshold level of body temperature that increases the eHsp72 level, which is observed in the Hsp72 response of other tissues. Importantly, results from passively heated animals indicate that the body temperature elevation, accompanied by similar kinetics to that of the exercise itself, is insufficient to induce both intracellular and extracellular Hsp72 responses. On the other hand, result from CEx animals suggest that exercise stress alone does not impart sufficient stress to increase eHsp72. Results of the current study emphasize that the exercise stress must be combined with body temperature elevation to induce the eHsp72 response.

Experimental Model of This Study

This study was performed based on a precedent work by Hamilton et al. (9), who showed that exercise-induced Hsps were blocked by preventing the elevation of body temperature. In our study, the 4°C condition did not elevate the colonic temperature in CEx animals by exercise (Fig. 1); consequently, Hsp72 in CEx animals did not increase significantly in the liver.
and skeletal muscle, although those of WEx animals increased significantly (Fig. 3). Therefore, these results indicate that our experiment might follow the conditions of the previous study (9). Aside from the parameters of Hamilton et al. (9), we measured the hemoglobin concentration and hematocrit as an index of the dehydration states of the animals. Consequently, no difference is apparent in these parameters among the three groups (Table 1). Therefore, our data of eHsp72 are inferred to be unaffected by hemococentration.

CEx animals failed to increase the eHsp72 by suppressing the elevation of body temperature during exercise in this study. However, this result does not rule out the possibility that exercise itself can increase eHsp72 without elevation of body temperature. In the case of the intracellular level, for example, cells exposed to a second heat shock after the first heat shock result in a reduction of Hsp72 production (22). Similarly, the Hsp72 levels in the biceps brachii muscle of well-trained athletes were attenuated after a resistance training program (7). Although it remains unclear whether the extracellular and intracellular stress responses are similar or not, results of these studies suggest that exercise-increased eHsp72 responses might also be blunted by repetitive exercise for nine days in this study. Considering that possibility, this study can demonstrate the influences of body temperature elevation on exercise-increased e-hsp72, but these results might not be able to show the influences of exercise on eHsp72 response.

Another concern is the possibility of nine days’ exercise increasing or decreasing the basal level of eHsp72 in WEx and CEx animals. In this regard, however, recent data by Yamada et al. (36) show that 10 days of exercise training in a 42°C environment did not change the basal level of serum eHsp72 in human subjects. Although the time frame during the 10 days was not examined, their study (36) suggests that short-term exercise training under the usual environment does not influence the basal eHsp72. Therefore, it is reasonable to assume that the basal eHsp72 level of exercise animals were not influenced by our exercise protocol and that the level was not different from that of CON level.

Finally, we might infer whether the electric shock influences the increase of eHsp72 during exercise or not because our control animals were not exposed to the electric shock during exercise. However, because CEx animals that received the electric shock did not increase eHsp72 after the exercise, it seems that the influences of electric shock on eHsp72 during exercise were not included in this study.

**Conclusions**

This study investigated the association between the elevation of body temperature and exercise-increased eHsp72 level. Our results suggest that the elevation of body temperature during exercise is an essential factor to induce the exercise-increased eHsp72 responses. Our results also propose that the possible role of body temperature elevation is displayed when the exercise stressor is combined with it. However, the relationship between body temperature and other factors to elicit exercise-increased eHsp72 remains unclear.

**Perspectives and Significance**

As a perspective of this study, to investigate whether various exercise adaptations change without having the exercise-increased eHsp72 is of interest. Indeed, it is suggested that eHsp72 functions on the immune system; therefore, the amount of eHsp72 induced by exercise might affect the adaptability of immunity. This topic should be addressed in a future study and our experimental model would be useful in examining it.

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