


Short-term treadmill exercise in a cold environment does not induce adrenal Hsp72 and Hsp25 expression

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Abstract Heat shock proteins (Hsps) have a critical role in maintaining cellular homeostasis and in protecting cells from a range of acute and chronic stressful conditions. Treadmill running exercise results in increased Hsp72 and Hsp25 levels in various tissues and heat production during exercise has been shown to be the main factor for the increased levels of Hsp72 in myocardium. Since the adrenal gland plays a vital role in general response to stress, regulation of Hsps in adrenal glands following stressful events seems to be critical for controlling the whole-body stress response appropriately. This study tested the hypothesis of whether elevation of temperature is solely responsible for exercise-induced adrenal Hsp72 and Hsp25 expression. Female Sprague–Dawley rats (3 months old) were randomly assigned to either a sedentary control group or one of two treadmill-running groups: a cold exercise group run in a cold room at 4 °C (CE), and a warm exercise group run at 25 °C temperature (WE). Animals were run 60 min a day at 30 m min⁻¹ speed for 4 consecutive days following adaptation to treadmill exercise. Exercise resulted in a significant elevation of body temperature only in the WE group ($p < 0.05$). Adrenal Hsp72 and Hsp25 levels were significantly higher in the WE group compare to the other groups ($p < 0.05$). These data demonstrated that exercise-related elevations of body temperature could be the only factor for the inductions of adrenal Hsp72 and Hsp25 expression.

Keywords Hsp72 · Hsp25 · Cold exercise · Adrenal · Treadmill running

Introduction

Sustaining of homeostasis from molecular to physiological level is important for survival of organisms under a variety of stressful conditions [27]. As part of both the sympathetic nervous system and hypothalamo–pituitary–adrenal axis (HPA), the adrenal gland plays a vital role in general response to stress. On the other hand, heat shock proteins (Hsps) have a critical role in maintaining cellular homeostasis and in protecting cells from a range of acute and chronic stressful conditions. Therefore, regulation of Hsps in adrenal glands following stressful events seems to be critical for controlling whole-body stress response appropriately.

It is well known that physiological stress created by treadmill exercise results in the common response of induction of Hsp72 in variety of tissues and organs including skeletal muscle, heart, liver, and brain [10, 22, 32]. In addition to raised temperature, several mechanisms including increased intracellular Ca²⁺ concentrations, metabolic overload, cellular energy depletion, hypoxia, and production of reactive oxygen species have been proposed for increased Hsp72 expression in response to exercise [31]. As opposed to these, considerable evidence suggests that tissue hyperthermia during exercise might be solely responsible for up-regulation of Hsp72 in the myocardium, since exercise-induced myocardial Hsp72 expression was abolished when hyperthermia was prevented in acute [36], short-term [16], and chronic treadmill-running exercise [17].

Chronic treadmill running, with a progressive rise in speed or grade, resulted in increased levels of Hsp72, the

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inducible member of the 70-kDa Hsp family, in the adrenal gland and these cellular stress responses increased as duration of the daily exercise [9]. On the other hand, the effects of treadmill running on adrenal levels of Hsp25, one of the stress-induced small Hsps [19], is not known. Although longer duration of exercise could have augmented effects of hyperthermia, it is unknown if increased temperature is accountable for the exercise-induced Hsps expression in the adrenal gland. Strong evidence exists about tissue-specific regulation of heat shock proteins [20, 38] and the adrenal gland seems to have one of the unique Hsp responses against stressful stimuli. For example, although restraint resulted in a similar amount of increase in Hsp72 levels in both the adrenal gland and aorta, the glucocorticoid dexamethasone resulted in attenuation of these increases only in the adrenal gland [38]. The induction of adrenal Hsp72 has been proposed to be mediated by adrenocorticotrophic hormone, since increased adrenal Hsp72 by restraint stress was abolished when ACTH secretion was inhibited, and restored after supplementing external ACTH in hypophysectomized rats [3]. Supporting of these, patients with adrenocortical tumors have shown low levels of both Hsp72 and Hsp27 (human homologue of rat hsp25) in the adrenal gland since a tumor secretes high quantities of cortisol, which in turn results in a reduction of ACTH [30]. In addition, adult mouse Hsp25 mRNA has been shown to localize primarily in the adrenal cortex, whose cells are known to be responsive to ACTH [40]. Exercise training has been shown to induce HPA and sympathoadrenal activation in an activity-specific and intensity-dependent manner [7, 29, 35]. Cumulatively, these observations suggest that Hsp responses of adrenal tissue against exercise stimuli may be different from that of myocardium. In that respect, the effects of short-term treadmill exercise on adrenal Hsps expression is not known, although it has been associated with increased serum levels of systemic stress markers of adrenal gland [4]. Therefore, short-term treadmill exercise gives a great opportunity to test tissue-specific regulations of Hsps expression. Particularly, the effect of non-hyperthermic exercise on adrenal Hsp induction is unknown, and this forms the rationale for the current study. Since it has been shown that myocardial Hsp expression was abolished when tissue hyperthermia was prevented during short-term treadmill running in female rats [16], we especially investigated the effects of short-term high-intensity exercise in a controlled environmental temperature at 4 °C on adrenal Hsp72 and Hsp25 induction.

Materials and methods

This experimental protocol was approved by the Jun-tendo University Animal Care and Use Committee for use of animals in scientific research and followed

guidelines of established by Physiological Society of Japan.

Animals

Female 3-month-old Sprague–Dawley rats were obtained from a licensed laboratory animal dealer (Japan SLC, Shizuoka, Japan). All animals were kept under standardized conditions of 12:12-h light–dark cycle (0900–2100) and temperature (23 °C) with freely available rat chow and water. After 2 weeks of acclimatization period for housing conditions, rats were assigned to sedentary control (C, $n = 8$) or one of the exercise groups based on willingness to run: treadmill running exercise at 25 °C (WE, $n = 9$), and exercise at 4 °C (CE, $n = 10$) groups. All exercise protocols were performed 2–4 h after the beginning of rats' nocturnal (dark) cycle.

Exercise training protocol

To examine if the effects of exercise-induced elevation of body temperature is responsible for adrenal Hsp72 and Hsp25 induction, WE and CE animals were exercised for 9 days on a motor-driven treadmill (KN-73; Natsume, Tokyo, Japan) in a climate-controlled room at 25 or 4 °C, respectively. As detailed previously [28], training intensity and duration for both groups were the same. Animals started to run on a treadmill at 20–25 m min⁻¹ without grade for 10 min. The duration was increased per day by reaching 50 min on day fifth. After that, the rats were trained for 4 consecutive days of treadmill exercise, 60 min/day, 30 m/min with 0 % grade. To cope with the effects of daily animal handling stress, control animals were put on a treadmill for 10 min/day without switching on the treadmill and after that they were placed beside the animal treadmill during WE group completing their daily running. Mild electrical shocks (30–40 V) were used sparingly for motivating the animals to run. Rectal temperatures were measured by using a calibrated thermistor probe (Shibaura Electronics, Tokyo, Japan) before and after each bout of daily exercise session. The exercise protocol used in this study was a minor modification of the protocols showing significant increase in Hsp72 in heart tissue [10].

Tissue isolation

Animals were killed with an intraperitoneal injection of 50 mg/kg pentobarbital sodium at the end of the last exercise session. Both adrenal glands were trimmed free of adhering fat and connective tissue and weighed. To identify tissue-related regulation of Hsps induction, the hearts were quickly removed and cleaned from blood with saline. Then,

tissues were frozen in liquid nitrogen and stored at $-85\text{ }^{\circ}\text{C}$ until Western-blot analysis.

Western-blot analysis

Adrenal glands and hearts were homogenized on ice in a buffer containing 10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA (pH 7.4), and protease inhibitor mixture (complete tablet; Roche Diagnostics, IN). Homogenates were centrifuged at $400 \times g$ for 15 min and supernatant was removed. Total protein concentrations were assessed spectrophotometrically using the Bradford assay. Individual protein samples (20 μg) were run on 10 % standard SDS-PAGE gels. Following electrophoretic separation, proteins were transferred to nitrocellulose membrane (0.45 μm , Bio-Rad) and blocked for 30 min using 5 % nonfat dry milk in Tris-buffered saline with 0.1 % Tween-20 (TBST). Then membranes were reacted with alkaline phosphatase-conjugated monoclonal antibody recognizes Hsp72 (SPA-810 AP, Stressgen) and anti-Hsp25 polyclonal antibody (SPA-801, Stressgen). Primary antibodies were diluted in a 1:5000 dilution in TBST with 2.5 % nonfat dry milk. Appropriate secondary antibody conjugated for alkaline phosphatase was used for Hsp25. Subsequent color development was performed using an alkaline phosphatase conjugate substrate kit (Bio-Rad). The abundance of Hsp72 and Hsp25 in each lane was normalized by using Coomassie staining of membranes for total protein determination. Quantification of the bands from the immunoblots was performed using computerized densitometry (ImageJ, ver. 10.2; National Institutes of Health, Bethesda, MD).

Statistical analysis

Results were expressed as the mean \pm standard deviation (SD). Body weight, adrenal weight, and relative adrenal weight (adrenal weight/body weight) data were analyzed using one-way ANOVA with Tukey post hoc test for overall comparison among the multi-group mean. To detect

significant differences between initial and final body weight, measurements were analyzed by using paired-sample *t* test. Differences of the colonic temperature were analyzed using two-way ANOVA with Bonferroni correction. Significance was established at $p < 0.05$.

Results

Adrenal weight/body weight ratio

Body weight, adrenal weight, and adrenal weight to body weight ratios for each group are shown in Table 1. Short-term treadmill running did not have an effect on body weight ($F(2, 24) = 0.18, p > 0.05$) adrenal weight ($F(2, 24) = 2.83, p > 0.05$), and adrenal weight to body weight ratio ($F(2, 24) = 1.80, p > 0.05$) in both exercise training groups.

Exercise and colonic temperature

Figure 1 shows average colonic temperature of the animals before and after 60-min duration of the last four exercise sessions. Pre-exercise colonic temperatures of animals were not different among groups ($p > 0.05$). On the other hand, exercise resulted in elevation of colonic temperature only in the WE group ($F(2, 24) = 41.1, p < 0.001$) compared to both CE and C groups. The average post-exercise colonic temperature for the last 4 days (i.e., 60 min running for WE and CE groups) for WE, CE, and C groups were $40.5 \pm 0.3, 38.5 \pm 0.6, \text{ and } 38.1 \pm 0.2$, respectively.

Exercise-induced HSP72 and 25 expressions in heart and adrenal

A representative Western blot and the relative amount of both adrenal and myocardial Hsp72 and Hsp25 in control and two exercised groups are shown in Figs. 2 and 3, respectively. Exercise training resulted in an increased level of both adrenal and heart Hsp72 only in the WE group

Table 1 Animals' body weight, adrenal weight, and adrenal weight/body weight ratio

Groups	Body wt. (g)		Adrenal wt. (mg)	Adrenal wt./final body wt. (mg g^{-1})
	Initial	Final		
C	219.2 \pm 6.1	228.4 \pm 6.7*	31.9 \pm 2.0	0.140 \pm 0.011
WE	220.9 \pm 8.4	238.7 \pm 15.2*	36.9 \pm 6.5	0.154 \pm 0.022
CE	219.4 \pm 5.0	234.3 \pm 11.0*	35.8 \pm 3.5	0.153 \pm 0.015

Values are mean \pm SD, $n = 8$ for sedentary control (C), $n = 9$ for exercise at $25\text{ }^{\circ}\text{C}$ (WE) and $n = 9$ for exercise at $4\text{ }^{\circ}\text{C}$ (CE) groups

Data were analyzed by one-way ANOVA for more than one comparison

Initial and final measurements of body weight were analyzed by paired-sample *t* test

* $p < 0.05$ difference between initial and final body weight

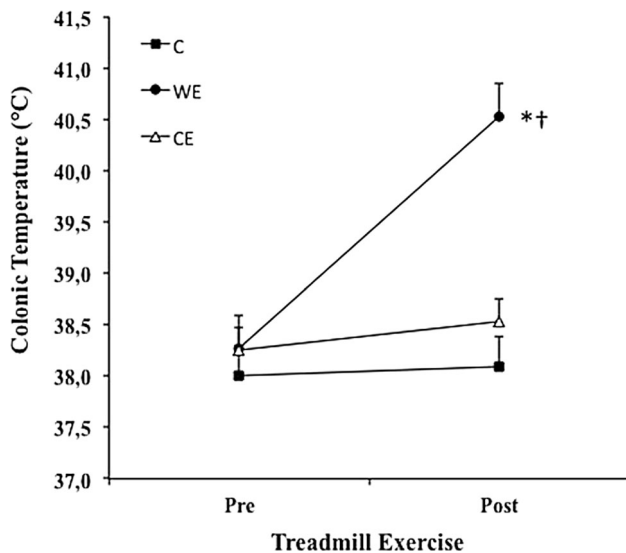


Fig. 1 Average colonic temperature of groups of animals. Colonic temperatures were obtained before and after each 60 min of exercise sessions for last 4 consecutive days. Values are mean \pm SD, $n = 8$ for sedentary control (C), $n = 10$ for exercise at 25 °C (WE), and $n = 9$ for exercise at 4 °C (CE) groups. * $p < 0.001$ compared to before exercise in the same group, † $p < 0.001$ WE versus both C and CE after exercise

($p < 0.05$). Similarly, the induction of adrenal and heart Hsp25 increased only in warm exercised animals ($p < 0.05$). Further, percentage increase in Hsp72 and Hsp25 were comparable in both heart and adrenal following short-term exercise in the WE group.

Discussion

Overview of principal findings

In this study, we asked a question if short-term exercise training results in an increase of adrenal Hsp72 and Hsp25 levels. In addition, we specially investigated if we prevent an exercise-induced increase in colonic temperature by training animals in a controlled environmental temperature at 4 °C, would adrenal gland still have the same Hsp response. Along with increased levels of Hsp72, these data have for the first time shown that short-term exercise resulted in an elevation of Hsp25 levels in rat adrenal glands. Furthermore, our data clearly indicate that hyperthermia is required for exercise-induced Hsp up-regulation in adrenal gland. Brief discussion of our findings is as follows.

Heat shock proteins are a family of highly conserved proteins found in all organisms, from bacteria to humans [24]. The family of Hsps, including Hsp72, an inducible form of 70-kDa Hsps and Hsp25, one of the stress-

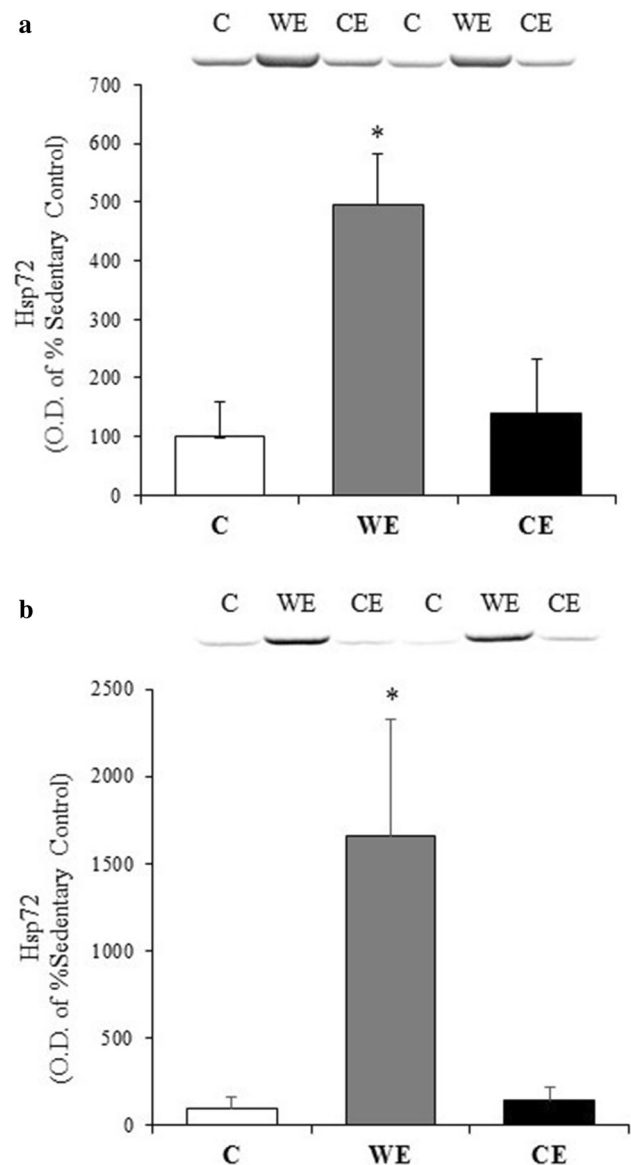


Fig. 2 Representative Western blot including two samples from each group and densitometric scanning of Hsp72. Immunoblot analysis shown in both adrenal gland (a) and heart (b). Results are expressed as the optical density unit (O.D. mean \pm SD of % sedentary control). Values are mean \pm SD, $n = 8$ for sedentary control (C), $n = 10$ for exercise at 25 °C (WE) and $n = 9$ for exercise at 4 °C (CE) groups. * $p < 0.05$ WE versus both C and CE at the end of the last exercise session

inducible small Hsps, is considered an endogenous defense system that enhances the survival of mammalian cells exposed to various types of stressful situations [18]. In addition to raised temperature, a wide selection of stressful stimuli has been shown to result in a marked increase in Hsp synthesis [24].

The adrenal gland plays a vital role in both short-term and long-term stress responses of an organism by releasing catecholamines and corticosteroids. In addition to fight-or-

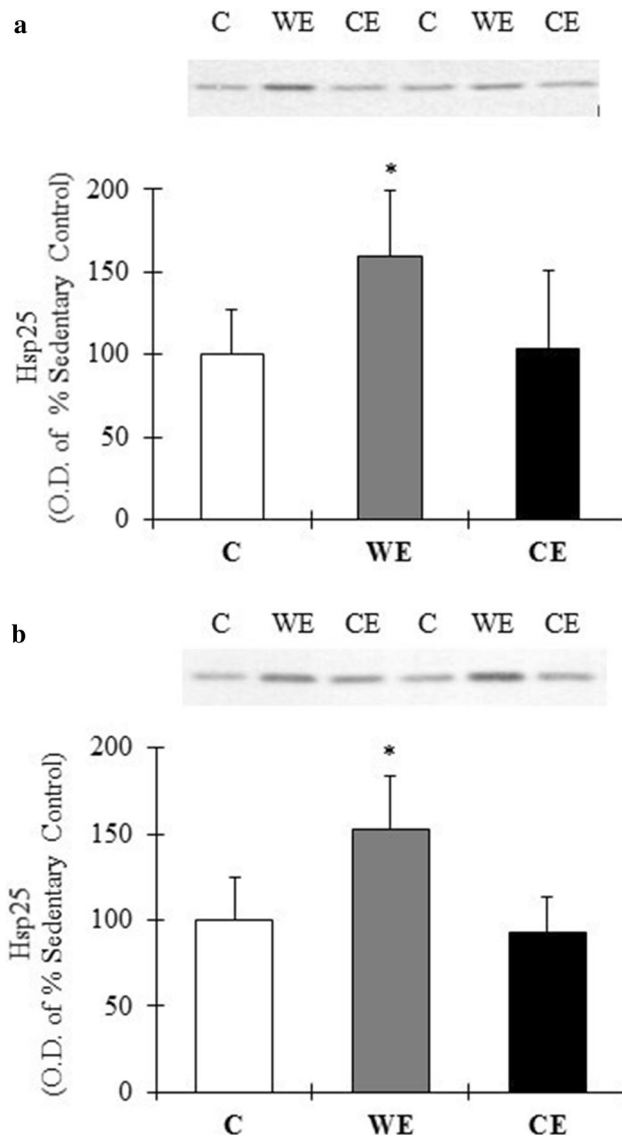


Fig. 3 Representative Western blot including two samples from each group and densitometric scanning of Hsp25. Immunoblot analysis shown in both adrenal gland (**a**) and heart (**b**). Results are expressed as the optical density unit (O.D. mean \pm SD of % sedentary control). Values are mean \pm SD, $n = 8$ for sedentary control (C), $n = 10$ for exercise at 25 °C (WE) and $n = 9$ for exercise at 4 °C (CE) groups. * $p < 0.05$ WE versus both C and CE at the end of the last exercise session

flight response and glucose metabolism, adrenal hormones may play a role in the regulation of Hsps in other tissues.

For example, adrenalectomy abolished cat exposure induced intracellular Hsp72 induction in various regions of brain [13]. Therefore, regulation of protective molecular chaperones in adrenal glands following stressful events seems to be critical for controlling the whole-body stress response appropriately. Indeed, high basal concentration of adrenal Hsp72 has been reported [6]. In addition, adrenal gland has been stated to be very sensitive to the type of

stressors ensuing the Hsp72 reaction [3]. Studies have shown that various stresses such as restraining [3], tail shock [6], chronic treadmill exercise [9], and acute exhaustive running [6] resulted in increased levels of Hsp72 in adrenal gland. Although the expression of Hsp25 in adrenal gland has not been reported, it could be induced by exercise training in skeletal muscle [15] and heart [8]. Therefore, we have hypothesized that short-term treadmill exercise would result in elevated levels of adrenal Hsp72 and Hsp25 even without an increase in core temperature, by means of exercising in a temperature-controlled environment. The present study shows that treadmill running resulted in elevated levels of both Hsp72 and Hsp25 in adrenal gland. On the other hand, our study has clearly shown that preventing hyperthermia inhibits exercise-induced elevation of adrenal Hsp72 and Hsp25 levels, since an increase in both Hsp72 and Hsp25 were only apparent in the group exercised at 25 °C temperature.

The induction of adrenal Hsp72 has been proposed to be mediated by adrenocorticotropic hormone, since increased adrenal Hsp72 by restraint stress is abolished when ACTH secretion is inhibited, and restored after supplementing external ACTH in hypophysectomized rats [3]. In addition, patients who have reduced plasma ACTH concentration because of adrenocortical tumors secreting high quantities of cortisol have shown low levels of both Hsp72 and Hsp27 (human homologue of rat hsp25) in adrenal gland [30]. On the other hand, both in vivo heat stress and exercise training have been shown to elevate plasma levels of ACTH [33]. Evidence shows that the hypothalamus–pituitary–adrenocortical axis, as well as the sympatho-adrenomedullary system, is mainly involved in mediating the physiological effects of physical exercise [35]. Indeed, 8 weeks of daily low-intensity treadmill exercise resulted in an approximate 10 % increase in adrenal weight while the increase was 31 % at high-intensity running in mice [2]. Fourteen days of variable chronic stress resulted in adrenal enlargement and augmented maximal adrenal responses to ACTH [39]. In our study, we did not measure the adrenal hormones or ACTH levels. In addition, adrenal weight or adrenal weight-to-body-weight ratio were not significantly different among groups. Therefore, increases in Hsp72 and Hsp25 levels in the WE group can not be associated with ACTH levels or involvement of the hypothalamus–pituitary–adrenocortical axis. On the other hand, a similar trend to increase in both adrenal weight and adrenal weight-to-body weight ratio existed in both exercise groups. Indeed, increased amounts of both catecholamine release and cortisol secretion have been reported during exercise in cold temperatures compared to the same exercise in warm temperatures in humans [37]. Therefore, we may assume that despite having comparable levels of stress, exercise in a controlled-environmental condition by

means of blocking increase in colonic temperature prevented induction of both Hsp72 and Hsp25 levels in adrenal gland.

Both whole-body heat stress and exercise have been shown to induce Hsps in various tissues in rats and other mammals. Hsp72 levels have been shown to increase with an elevation of the environmental temperature in mice liver exposed to 40, 42, 44, and 46 °C whole-body heat stress [23]. It has been previously shown that even 3–5 days of short-term exercise training results in increased Hsp72 levels in rat myocardium [10]. Besides elevation in body temperature, other similarities exist between heat stress and exercise-related changes in the cellular milieu, which may result in Hsp up-regulation; such as decrease in intracellular pH, increase in lactate formation and cytosolic calcium levels, and production of reactive oxygen species [5, 11, 12, 26, 32, 37, 41]. Therefore, it has been stated that hyperthermia is not the merely factor for increased Hsp induction [25]. Indeed, in one of the early studies, acute submaximal treadmill running at cold environment (14 °C) resulted in increased levels of Hsp72 in the left ventricle without having any increase in colonic temperature (i.e., about 38 °C). Although elevated left ventricular Hsp72 have been seen in untrained rats when core body temperature was kept similar to warm exercised animals (about 41 °C), the highest level of Hsp72 was seen in warm exercised group. Therefore, authors have concluded that factors in addition to heat stress may contribute to induction of Hsp72 during exercise [34]. While the temperature of the room for the cold exercise group during running was lower in our study (i.e., 14 vs. 4 °C) the colonic temperature at the end of the each exercise session was comparable in both studies (i.e., about 38 °C). Despite the fact that the intensity and volume of treadmill exercise was much higher in our study, we have found that elevated core temperature is required for exercise-induced increase in left ventricular Hsp72. Supporting our findings, when tissue hyperthermia is disallowed by using cold environment during short-term [16] treadmill running exercise, up-regulation of Hsp72 in the myocardium was prevented. We have further carried out that finding by showing that hyperthermia is required also for myocardial Hsp25 induction following short-term treadmill running.

Recently, it has been reported that rats running at 25 °C have higher internal and hypothalamic temperature compared to rats running at 12 °C [14]. In addition, time to fatigue was longer in rats running under the effects of external cooling [14]. Despite the fact that CE and WE groups of rats run at the same speed and duration in our study, WE group rats most likely expended more effort than that of CE group that may be contributed to increased adrenal Hsp72 and Hsp25 levels.

Critique of experimental model and limitations of study

Although sexual differences have been shown in ACTH and stress hormone release in the adrenal gland [1], the adult female Sprague–Dawley rat was chosen as an experimental model because, (1) It has been shown that female rodents have better running performance in both speed and endurance compared to the male counterparts [21], (2) Studies displaying increased Hsp72 levels in ventricle [10, 16], and revealing abolishment of myocardial Hsp72 expression when hyperthermia was prevented following similar short-term running exercise were used female Sprague–Dawley rats [16].

As a conclusion, despite the reported sensitivity of adrenal tissue to type of stressor inducing Hsp responses, our data clearly showed that hyperthermia is required for exercise-induced induction in Hsp72 and Hsp25 levels in adrenal tissue.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest. The authors would like to thank Dr. Tahir Hazir for his statistical assistance.

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