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

Senay Akin1 • Hisashi Naito2 • Yuji Ogura2 • Noriko Ichinoseki-Sekine2 • Mitsutoshi Kurosaka2 • Ryo Kakigi2 • Haydar A. Demirel1

Received: 18 March 2016 / Accepted: 11 July 2016 / Published online: 28 July 2016
© The Physiological Society of Japan and Springer Japan 2016

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 compared 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
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 adrenocorticotropin 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 Juntendo 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,
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 × 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

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 ± 0.3, 38.5 ± 0.6, and 38.1 ± 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 ± 6.1 | 228.4 ± 6.7*    | 31.9 ± 2.0                      | 0.140 ± 0.011 |
| WE     | 220.9 ± 8.4 | 238.7 ± 15.2*   | 36.9 ± 6.5                      | 0.154 ± 0.022 |
| CE     | 219.4 ± 5.0 | 234.3 ± 11.0*   | 35.8 ± 3.5                      | 0.153 ± 0.015 |

Values are mean ± SD, n = 8 for sedentary control (C), n = 9 for exercise at 25 °C (WE) and n = 9 for exercise at 4 °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
(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-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-
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-adreno-medullary 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 adrenals 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
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.

Grants

This study was supported by Juntendo University Institute of Health and Sports Science & Medicine. This study was also supported by a Grant-in-Aid for Scientific Research (16300212 and 12480011 to H. Naito) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of 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.

References

1. Babb JA, Masini CV, Day HE, Campeau S (2013) Sex differences in activated CRF neurons within stress-related neurocircuitry and HPA axis hormones following restraint in rats. Neuroscience 234:40–52
2. Bartalucci A, Ferrucci M, Pulcheri F, Lazzeri G, Lenzi P, Toti L, Serpillette FR, La Torre A, Gesi M (2012) High-intensity exercise training produces morphological and biochemical changes in adrenal gland of mice. Histol Histopathol 27(6):753–769
3. Blake MJ, Udelsman R, Feulner GJ, Norton DD, Holbrook NJ (1991) Stress-induced heat shock protein 70 expression in adrenal cortex: an adrenocorticotropic hormone-sensitive, age-dependent response. Proc Natl Acad Sci USA 88:9873–9877
4. Brown DA, Johnson MS, Armstrong CJ, Lynch JM, Caruso NM, Eihlers LB, Fleschner M, Spencer RL, Moore RL (2007) Short-term treadmill running in the rat: what kind of stressor is it? J Appl Physiol 103:1979–1985
5. Burdon RH, Kerr SM, Cutmore CM, Munro J, Gill V (1984) Hyperthermia, Na+ K+ ATPase and lactic acid production in some human tumour cells. Br J Cancer 49(4):437–445
6. Campisi J, Leem TH, Greenwood BN, Hansen MK, Moraska A, Higgins K, Smith TP, Fleschner M (2003) Habitual physical activity facilitates stress-induced HSP72 induction in brain, peripheral, and immune tissues. Am J Physiol Regul Integr Comp Physiol 284:R520–R530
