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Activation of HPA Axis and Remodeling of Body Chemical Composition in Response to an Intense and Exhaustive Exercise in C57BL/6 Mice

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Summary

Several deleterious effects may occur when intense and exhaustive exercise (IE) is not well-planned. This study aimed to investigate the effects of a short duration IE on body chemical composition and hypothalamic-pituitary-adrenal (HPA) axis. C57Bl/6 mice were distributed into four groups (10 mice per group): control (C-4D and C-10D), 4 days (E-4D), and 10 days of IE (E-10D). IE program consisted of a daily running session at 85 % of maximum speed until the animal reached exhaustion. Body weight as well as total body water, fat and protein content were determined from animal carcasses. HPA activation was assessed by plasma corticosterone levels measured by radioimmunoassay and the weight of both the adrenal glands and thymus were measured. Plasma corticosterone levels increased by 64 % in both the E-4D and E-10D groups. The weight of the adrenal glands augmented by 74 % and 45 %, at 4 and 10 days of IE, respectively, whereas thymus weight diminished by 15 % only in the E-10D group. The total carcass fat content decreased by 20 % only at 4 days IE, whereas protein content decreased by 20 % in both E-4D and E-10D groups. A relationship between corticosterone plasma levels and loss of body protein content in both E-4D and E-10D groups was observed (R^2=0.999). We concluded that IE may be related to HPA axis activation associated with remodeling of body chemical composition in C57BL/6 mice.

Key words

Intense exercise • HPA axis • Body composition adrenal glands • Thymus • Gastrocnemius muscle

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Introduction

Physical activity and exercise training programs are strongly associated with health, fitness and quality of life (Fiocco *et al.* 2013), by exerting beneficial effects on health and preventing chronic diseases. In fact, exercise contributes to delaying chronic-degenerative diseases (Chakravarthy *et al.* 2002), aging (Rosa *et al.* 2005), osteoporosis, atherosclerosis, sarcopenia (Chakravarthy *et al.* 2002) and diabetes (Zinman *et al.* 2003).

A combination of elevated physical activity and caloric restriction is the recommended strategy for controlling body weight gain. Studies on the long-term effectiveness of physical exercise/activity with or without diet and/or behavioral modification therapy in the control of body weight gain conclude that training intensity should be moderate (Sodlerlund *et al.* 2009). However, resistance exercise training is gaining wider acceptance as a supplement to endurance exercise training in the control of cardiovascular risks, particularly in diabetes and obesity (Tresierras and Balady 2009). In contrast to the well-known beneficial effects of endurance and resistance exercise (Konig *et al.* 2001), much less is known on the effects of short-term, intense and exhaustive exercise (IE)

on body weight control and/or body chemical composition remodeling.

Regulation and synthesis of the hormones in general are controlled and/or modulated by the hypothalamus-pituitary-adrenal axis (HPA axis), which in turn is sensitive to physical and/or emotional stress. These two conditions are usually observed in exercise. Accordingly, exercise stimulates the hypothalamus to release corticotrophin releasing hormone (CRH), which in turn elicits adrenocorticotropin hormone (ACTH) release, leading to secretion of corticosteroid hormones by the adrenal cortex (Fevold 1967). While in humans the most abundant stress hormone is cortisol (Staehelin *et al.* 1955), corticosterone is the main stress hormone in rodent species (Levine and Treiman 1964).

The influence of exercise on the HPA axis was first reported in 1955 by Staehelin and colleagues. These authors demonstrated an increase in the plasma cortisol concentration throughout a 2 h-cycling exercise, followed by a decrease back to basal level immediately after the end of the exercise bout. The cortisol response has been shown to be dependent on several factors, such as exercise intensity and metabolic pathways (anaerobic or aerobic), physical pre-conditioning, and previous nutritional procedures (Brandenberger and Follenius 1975).

Unraveling the effects of different types of exercise program on various tissues and organs, as well as their role in regulating body chemical composition and weight may contribute to a better knowledge on the use of exercise to combat the adverse effects of chronic-related diseases such as diabetes and obesity. Thus, there is great interest in developing alternative and non invasive strategies in the fight against obesity and associated co-morbidities.

High intensity exercise is usually associated with fatigue and burnout (Horstman *et al.* 1979, Konig *et al.* 2001). However, studies concerning the contribution of this type of exercise to body chemical composition are still scarce. We previously reported that both cognitive (Rosa *et al.* 2007) and intestinal functions (Rosa *et al.* 2008) are sensitive to an intense and exhaustive exercise (IE) program. Indeed, it is clear that this type of exercise triggers important physiological responses which are not yet fully understood. Thus, the aim of the present study was to further investigate the effects of IE on body chemical composition (water, fat and protein content, gastrocnemius cross-sectional area) and to ascertain its possible relationship with exercise-induced HPA axis activation in a rodent animal model (C57BL/6 mice).

Methods

Animals

Inbred male C57BL/6 mice (3 month old, 27±2 g) were obtained from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia-Universidade Federal de São Paulo (CEDEME) and housed in groups of five animals per cage with water and food (Mice Chow – Nuvilab) *ad libitum*. Animals were kept on a 12:12 h light-dark cycle (06:00 to 18:00 h) and maintained at 23±1 °C for at least 5 days before the beginning of experiments and throughout the experimental period. Animals were randomly assigned to four groups (10 animals per group): animals submitted to either 4 days (E-4D) or 10 days of IE (E-10D), and their corresponding 4 and 10-day control groups (sedentary animals). Food intake per animal was calculated by subtracting the food leftover per cage per day from the daily food quantity offered, and was expressed as mean food quantity per animal. Change in animal body weight was calculated by subtracting final weight from initial weight. All body weight measurements were done at 17:00 h. Animals were sacrificed by decapitation 10 min after the end of the last exercise session, together with their corresponding control (C-4D and C-10D) groups. The gastrocnemius muscle was carefully isolated for histological analysis. The thymus and adrenal glands were quickly removed and weighed (OHAUS, AS200). Animal handling procedures were approved by the University's Ethics Committee in compliance with the International Guiding Principles for Biomedical Research Involving Animals (CIOMS 1985).

Exercise protocol

Animals were submitted to an IE program, previously described in Rosa *et al.* (2007), which we have previously described as being effective to increase plasma lactate concentration, decrease physical performance, and enhance skeletal oxidative stress. Briefly, all animals were submitted to an adaptation period to a motor-driven treadmill (Exer 3/6 Columbus Instruments, Columbus, OH, USA) environment. After determination of maximum running velocity (V_{max}) for each animal, by means of maximal incremental test according to Rosa *et al.* (2005), the exercised animal groups performed a daily bout of IE for either 4 or 10 consecutive days, consisting of: 1) 3-min warm-up at 5 m/min, 2) running until

exhaustion at 85 % of maximum velocity, and 3) 3-min cool down at 5 m/min. Treadmill grade was set at 0 % for all exercise bouts, which were performed between 16:00 and 18:00 h. After the exercise session, animals were placed back in their home-cages. Mice were stimulated to run by gentle hand prodding with a soft brush throughout the course of the exercise program. Animal exhaustion was identified by refusal of the animal to run or failure to keeping pace with treadmill speed even after gentle stimulation. C-4D and C-10D animals were exposed to the treadmill environmental conditions, such as handling, motor noise, vibration, and deprivation of food and water, except for the exercise session.

Corticosterone levels

Total blood was collected in cooled plastic tubes containing 0.1 ml of EDTA (60 mg/ml), and centrifuged at 2300 rpm for 20 min at 4 °C. Plasma from each animal was collected in polycarbonate tubes and stored in a freezer at −20 °C for further analysis. Corticosterone levels were determined by a rodent specific radioimmunoassay method using a commercial kit (ICN Biomedicals, Costa Mesa, CA, USA), adapted from Thrivikraman *et al.* (1997). The assay sensitivity was 3.125 ng/ml, and inter assay and intra assay variations were 10.3 % and 7.1 %, respectively.

Body chemical composition

After animal exsanguination, the abdominal cavity was opened, the gut removed and carefully emptied of its content, and replaced within the animal carcass. The carcass was weighed and homogenized in an equal volume of water. Two samples (4 g) of the homogenized material were stored at 4 °C for further fat and protein content analysis. The remaining homogenized material was dried to constant weight in an oven at 60 °C, and a sample of the resulting powder was burnt in an adiabatic calorimeter (IKA C-5000). The water content was calculated by the difference between the weight of the wet and the dry carcasses for each animal, and expressed as a percentage of carcass weight. Fat content was measured in fresh samples of homogenized carcasses according to the chloroform-methanol method (Folch *et al.* 1957). Protein content was determined according to the Lowry method (Leshner *et al.* 1972).

Gastrocnemius histological studies

Fresh tissue samples from all animal groups were obtained and appropriately stained with hematoxylin and eosin technique. Briefly, gastrocnemius muscle samples were fixed in 10 % buffered formalin, dehydrated by sequential exposure to graded concentrations (from 50 to 85 %) of ethyl alcohol, cleared in four rinses of xylene, embedded in paraffin wax at 58.0±0.5 °C, and sectioned into 4 µm-thick transversal slices. Muscle fibers cross-sectional area was quantified from light micrographies (10x and 40x lens) by blind evaluation of the cross-sectional area of 15 randomly individual fibers from 4-spot per tissue sample for each animal in all animal groups. Tissue samples were analyzed by computer software (Image Tool 3.00 for Windows, Health Science Center, University of Texas, San Antonio, USA).

Chemicals

All chemicals were analytical grade. Salts, ethyl alcohol, acetic acid, formaldehyde, and xylene were purchased from Merck (Darmstadt, Germany); hematoxylin and eosin were from Nuclear (Diadema, Brazil), and Corticosterone Radioimmunoassy Kit, from ICN Biomedicals (Costa Mesa, CA, USA).

Statistical analysis

Data were expressed as means ± standard error of the mean, with *n* representing the number of animals. Statistical significance was analyzed by two-way ANOVA, and Bonferroni's post-test. P values lower than 0.05 were considered statistically significant.

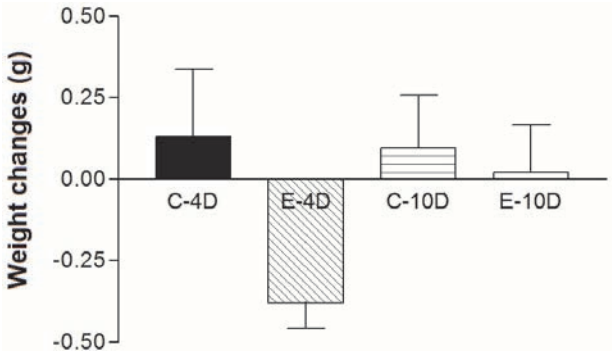

Fig. 1. Change in body weight of control (C-4D, n=10; C-10D, n=10), four-day exercised (E-4D, n=10), and ten-day exercised (E-10D, n=10) animals.

Results

Body chemical composition

Figure 1 illustrates the effects of 4 and 10 days of IE on animal body weight. As shown, IE caused a

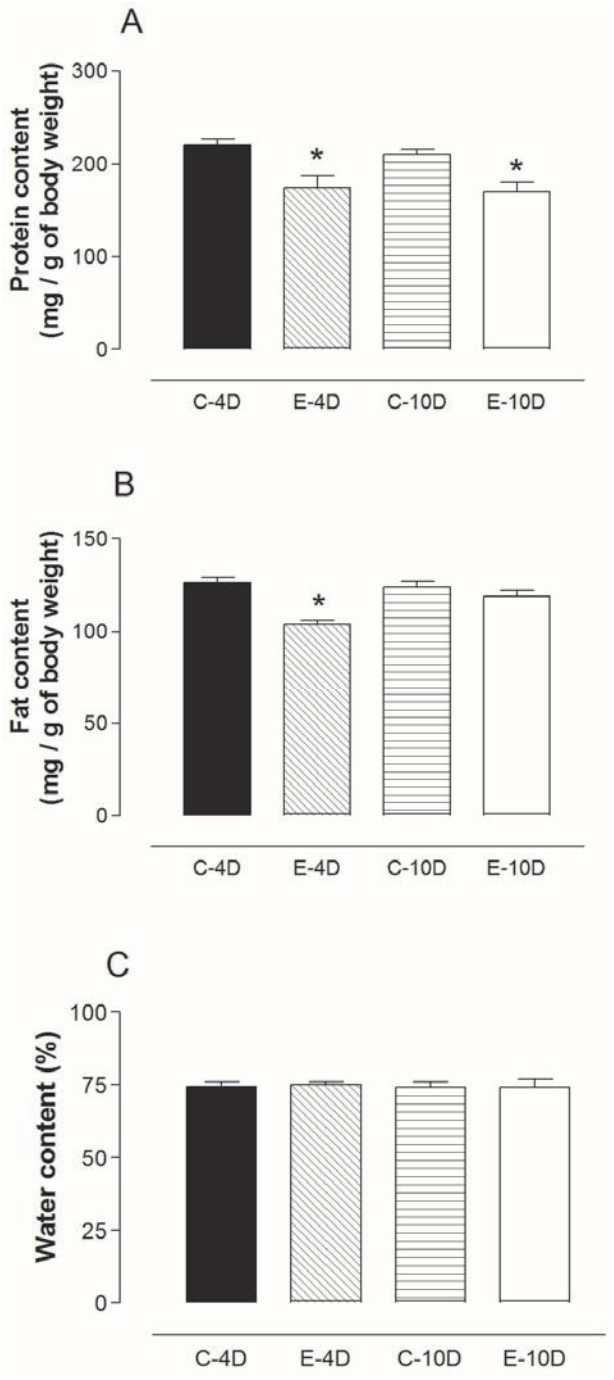

Fig. 2. Effect of IE on body chemical composition of C-4D (n=10), C-10D (n=10), four-day exercised (E-4D, n=10), and ten-day exercised (E-10D, n=10) animals. Levels of protein (**A**), fat (**B**) and water (**C**) content, respectively. * indicates significant difference (P<0.05) in comparison with correspondent control group.

significant loss (−0.38±0.08 g) in body weight on group E-4D, compared with the corresponding control group and with E-10D group, both of which showed no change (0.1±0.2 g and 0.02±0.12 g, respectively). There was no statistical difference in body weight between C-4D and C-10D groups (p=0.12). Similarly, there were no differences in the animal food intake between IE and their

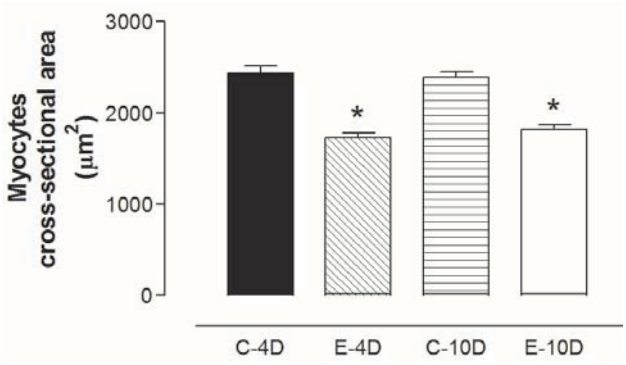

Fig. 3. Effect of IE on the cross-sectional area of gastrocnemius myocytes of C-4D (n=10), C-10D (n=10), four-day exercised (E-4D, n=10), and ten-day exercised (E-10D, n=10) animals. * indicates significant difference (P<0.05) in comparison with correspondent control group.

corresponding control groups, which averaged between 4 and 6 g/animal per cage.

Body fat content was significantly reduced by 20 % (P<0.001) in E-4D, as compared with the corresponding C-4 animal group (Fig. 2B, P<0.001). No differences in the body fat content were detected between E-10D and C-10D groups (P>0.05). Body protein content significantly decreased from 220±6 mg/g (C-4D animals) to 174±13 mg/g of body weight in E-4D animals (P<0.01), and from 210±5 mg/g (C-10D animals) to 170±10 mg/g (P<0.05) of body weight in E-10D animals (Fig. 2A), which resulted in 20 % body weight reduction in both groups. No differences in body protein content were observed between C-4D and C-10D animal groups (P>0.05). Likewise, body water content remained unaltered, at around 74 %, in all groups (Fig. 2C).

Histological studies

Figure 3 illustrates a significant reduction in the cross-sectional area of skeletal muscle fibers of the gastrocnemius muscle in response to IE (Fig. 3). The myocyte cross-sectional areas in both the E-4D and the E-10D groups were 30 % lower than in their respective control groups (P<0.001, for both comparisons).

Stress evaluation

The activation of HPA axis in response to IE were evaluated based on three stress markers: plasma corticosterone levels (Fig. 4), thymus and adrenal glands weights (Fig. 5). Similar increases (~64 %) in corticosterone plasma concentrations were observed in both E-4D (146±7 ng/ml) and E-10D (140±4 ng/ml) animals compared with their respective control groups (89±6 ng/ml in C-4D (P<0.001) and to 85±5 ng/ml in

C-10D (P<0.001); Fig. 4). No differences were observed between the C-4D and C-10D groups (P=0.14). The relative weight of the adrenal glands significantly increased from 0.019±0.002 % in the C-4D group to 0.033±0.004 % in the E-4D group (P<0.001), and from 0.020±0.001 % in the C-10D group to 0.029±0.001 % in the E-10D group (P<0.05; Fig. 5A). Finally, a significant reduction of 25 % in the thymus weight was detected only in the E-10D group (0.196±0.006 % of body weight) in comparison with the C-10D group (0.26±0.01 % of body weight) (P<0.03; Fig. 5B). No differences in thymus weight were seen between C-4D and C-10D groups (P=0.4), and between C-4D and E-4D groups (P=0.09).

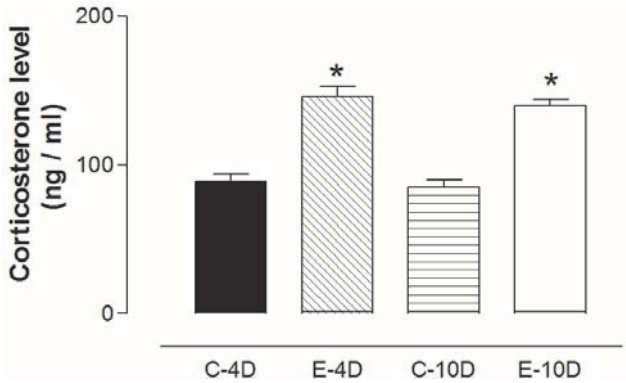

Fig. 4. Plasma corticosterone level at rest (C-4D, n=10; C-10D, n=10), immediately after the fourth day (E-4D, n=10), and the tenth day (E-10D, n=10) of exercise. * indicates significant difference (P<0.05) in comparison with control values.

Discussion

We have previously shown that the present treadmill running exercise program (IE) causes a significant reduction in the C57BL/6 mice's physical performance, which is accompanied by increased plasma lactate concentration to levels higher than 6 mM after both 4 and 10 days, and an exponential decrease in the time to animal exhaustion, thus characterizing the IE program as a very intense and exhaustive exercise (Rosa *et al.* 2007). In the present study we further corroborate this conclusion by demonstrating an important skeletal muscle damage evidenced by 30 % reduction in the fiber cross-section area of the gastrocnemius muscle (Fig. 3) after both 4 and 10 days of IE. It should be emphasized that the fiber type was not considered in the analysis, as the effect of intense exercise on skeletal muscle are well-known even though it is quite likely that the effects of IE would be more compelling on the fast IIb fibers due to the intensity of the exercise performed. However, it was out of the scope of the present study to characterize the effects of IE on the fiber types of the gastrocnemius muscle, but just to confirm the expected effects on this specific muscle. On the other hand, almost nothing is known about the effects of short-term exhaustive exercises on protein metabolism in skeletal muscle (Haus *et al.* 2007, Egan *et al.* 2013), and still less on the effects of this exercise type on body chemical composition remodeling.

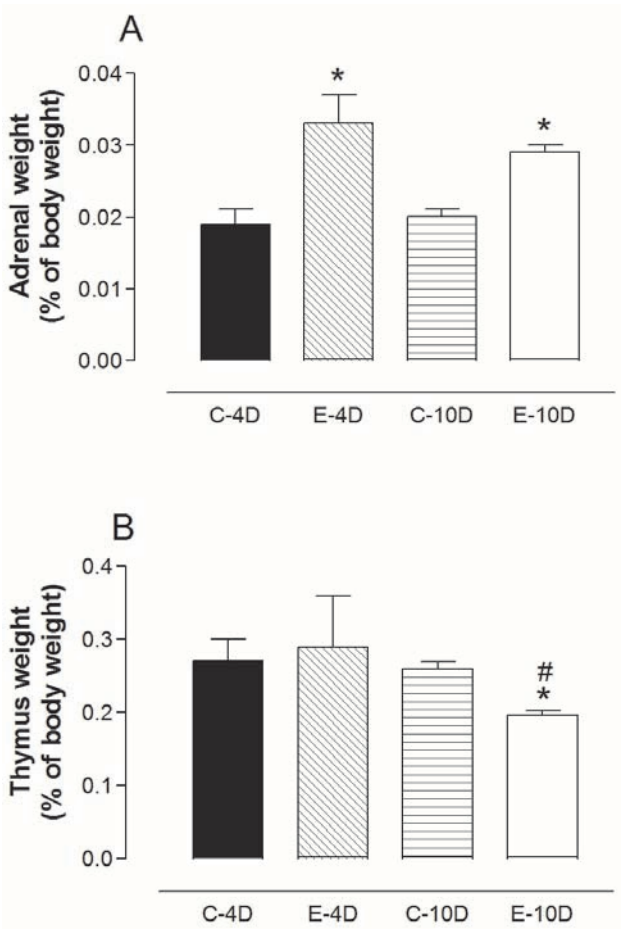

Fig. 5. Weight of adrenal (**A**) and thymus (**B**) glands, expressed as percentage of body weight in C-4D, C-10D, four-day exercised (E-4D), and ten-day exercised (E-10D) animal groups (n=10 for the four groups). * indicates significant difference (P<0.05) in comparison with correspondent control group and # in comparison to E-4D group.

Quite interestingly and surprisingly, IE program caused both body weight loss and body chemical composition remodeling. Indeed, there was a dramatic reduction in both body lipid content (20 %) and body protein content (30 %) after 4 days of IE (Fig. 2), although no changes in animal food intake were observed. However, these changes were transient, since

they are similar as those from the control animals after 10 days of IE (Fig. 1), even though exercise related intensity markers (plasma lactate concentration, animal time to exhaustion, and physical performance) indicates the maintenance of exercise-induced stress condition (Rosa *et al.* 2007). A cycling nature of body weight and body lipid composition in mice has been related to altered pattern of food efficiency when animals are exposed to novel environment (Wainwright *et al.* 1991). This seems to be the case here as we have previously shown that IE program induces distinct physiological conditions. In fact, either 4 or 10 days of IE causes distinct changes in the redox status and contractility of intestine and memory damages as well (Rosa *et al.* 2007, 2008). The cycling nature of body and weight remodeling may also be related to a gradual reduction in the body energy expenditure throughout the course of IE program, leading to a gradual decrease of the highly negative initial energy balance. Indeed, we have previously shown that daily exercise sessions, due to their high intensity feature (running at 85 % of V_{max}), rapidly leads to animal exhaustion, by reducing the exercise session from 186 s in the first day of treadmill exercise session down to 103 s and 53 s at 4 and 10 days of IE, respectively (for detail of the time course of time to exhaustion throughout the course of the IE program, see Rosa *et al.* 2007). Usually, body weight reduction in rodents takes place in response to long-term exhaustive exercise (Gomez-Merino *et al.* 2007), to long-term moderate exercise (Goto *et al.* 2007), or even after a short-term treadmill running (Brown *et al.* 2007), or with different types of exercise, such as swimming and voluntary wheel running (Ferrara *et al.* 1998). However, it has been shown that exercise together with controlled dietary intake rather than exercise alone is a better strategy in producing weight loss in different mice strains (Ouyang *et al.* 2010). Surprisingly, in the present study, both body weight and lipid content were reduced only at 4 days' IE, in the absence of any dietary restriction. Unfortunately, these reductions were not sustained in 10 days IE group, but this result may be explained by previous findings of reduced lipogenesis resulting from long-term exhaustive exercise training in rodent model (Gomez-Merino *et al.* 2007). Although we did not measure lipolysis, considering the exhaustive and fatiguing features of IE program, we might suppose that lipid mobilization was probably elevated above basal levels within the 24 h resting period in order to replenish the energy stores reduced and/or depleted during the exercise session. We may speculate of other possibilities leading to increased lipolysis, such as skeletal muscle damage-induced inflammation, which is known to increase IL-6 release from skeletal muscle (Pedersen 2007). Accordingly, in the present study, IE caused significant body protein degradation and atrophy in the muscle fibers of gastrocnemius muscle (Fig. 2C). Another possibility to explain increased lipolysis could be IE-induced activation of the enzyme adenosine monophosphate kinase (AMPK) (Winder 1998), known to increase both lipolysis and proteolysis to assure adequate body energy stores disposal. Finally, another possible explanation for the loss of body fat content observed at 4 days of IE yet not after 10 days (Fig. 2B), could be that short-term intense exercise increases plasma glucocorticoid levels, which in turn enhance lipolysis in adipocytes (Buono *et al*. 1986) leading to a redistribution of fat stores (McMurray and Hackney 2005). In fact, glucocorticoids mobilize subcutaneous fat tissue for readily energy production, whereas it drives the storage of visceral fat for liver substrate requirements (Dallman *et al*. 2007). Whatever mechanisms are responsible for both the body weight and chemical composition remodeling, the novelty of the present study is that lipid mobilization was observed with only 4-days of repetitive and very short-term daily exercise sessions (lower than 3 min), even though the cellular mechanisms underlying these changes remain an open question.

The observed elevation of lipolysis and proteolysis in response to IE (Fig. 2) strongly argue in favor of an exercise-induced stress response, which certainly involves activation of the HPA axis, and consequent increase in corticosterone secretion (Simmons *et al.* 1984). This certainly was the case, since corticosterone plasma levels at 4 and 10 days of IE (Fig. 4) were significantly higher than those in the CT group, in which the plasma corticosterone levels were within the ranges previously described for this animal strain (Jacobson *et al.* 2006). We thus confirmed the stressful effect of the present IE program, as it is known that cortisol/corticosterone levels are directly related to exercise intensity as a critical factor to trigger the enhancement of ACTH levels (Farrell *et al.* 1983, Buono *et al.* 1986). Moreover, it has been shown that only very intense running exercise, which increases plasma lactate concentrations above lactate threshold induces significant HPA axis activation (Soya *et al.* 2007). We now confirm that this is also true for C57BL/6 mice, which performed very intense and exhaustive treadmill running. In

addition, similar corticosterone levels at 4 days and 10 days of IE suggest the absence of stress habituation (Groves and Thompson 1970) (Fig. 4). Furthermore, the strong correlation between plasma corticosterone levels and protein degradation (R^2=0.999) suggests that IE program increases corticosterone secretion, probably associated with skeletal muscle proteolytic state (Simmons *et al.* 1984) and eventually body proteolysis. In fact, the role of skeletal muscle as an energy sensor and endocrine organ in the body has been recently postulated (Petersen 2013, Welc and Clanton 2013). Therefore, our data strongly argue in favor of IE program to represent an acute stress bout at every daily exercise session.

Another important issue that must be addressed in this study was whether this IE-induced stress response could become chronic. Two important markers of chronic stress response are adrenal glands hypertrophy and thymus involution, due to ACTH overstimulation. In the present study, we reported a remarkable hypertrophy of the adrenal glands induced by 4 or 10 days' IE program (Fig. 5), thus, corroborating the chronic stimulation of the HPA axis activation. Brown *et al.* (2007) reported that short-term treadmill running leads to chronic stress. However, there are two critical differences between their study and ours. Firstly, the animal gender and species were different (mice x rats); secondly, even though the duration of the exercise program was quite similar (up to 10 days of treadmill running), the whole exercise program was considerable different, as the daily exercise session in Brown's study was much longer (10 min warm-up at 15 m/min, 40 min running at 30 m/min, and 10 min cool-down at 15 m/min), than the duration of the exercise sessions in the present study, which were not longer than 3 min. In fact, the novelty of our data concerning this issue is that we got a remarkable adrenal glands hypertrophy with very small, but very intense, exercise session duration in contrast to the adrenal glands hypertrophy described by other authors, which always involves long-term exercise protocols (Ulrich-Lai *et al.* 2006). Corroborating this possibility, there was a 30 % reduction in the thymus weight in response to 10 days of IE (Fig. 5B), the second marker of chronic stimulation of HPA axis. Indeed, chronic stress was associated with cortisol-induced thymus involution (Raone *et al.* 2007), as cortisol is a potent immunologic suppressor (Roggero *et al.* 2006). Even though we did not address whether the thymus involution was due to apoptosis or necrosis in this study, thymus weight reduction by cell apoptosis in response to intense exercise has previously been demonstrated in C57BL/6 mice (Hoffman-Goetz *et al.* 1999).

In summary, to the best of our knowledge this is the first study to demonstrate a relationship between a very short-duration, intense and exhaustive exercise and chronic HPA axis activation, associated with remodeling of body weight and body chemical composition, strongly associated with body proteolysis. Even though we are proposing this relationship, our data do not rule out other possibilities, such as the involvement of catecholamines and inflammatory processes. Future studies are necessary to confirm our findings and to compare them with other exercises and models.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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