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High-intensity exercise training produces morphological and biochemical changes in adrenal gland of mice

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Summary. The effects of training are dependent on complex, adaptive changes which are induced by acute physical exercise at different levels. In particular, 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. The aim of the present study was to investigate, through a morphological and biochemical approach, the effects of training on the adrenal gland of mice, following two different protocols consisting of either low- or high-intensity training. Mice were run daily on a motorised treadmill for 8 weeks, at a velocity corresponding to 60% (low-intensity exercise) or 90% (high-intensity exercise) of the maximal running velocity previously determined by an incremental exercise test. We found that physical exercise produced an increase in the adrenal gland size compared with the control (sedentary) mice. The increase was 31.04% for mice that underwent high-intensity exercise and 10.08% for mice that underwent low intensity exercise, and this appeared to be the result of an increase in the area of both the adrenal cortex and adrenal medulla. Morphological analysis of the adrenal cortex showed that both types of exercise produced an increase in cytoplasmic vacuoles in steroidogenic cells, appearing more abundant after high-intensity exercise. No change was found in the reticulate zone. In the adrenal medulla, despite the absence of morphological changes, immunohistochemistry for tyrosine hydroxylase, dopamine ß-hydroxylase and phenyl-ethanolamine-N-methyltransferase demonstrated an increased immunopositivity for these cathecolamine-synthesizing enzymes after intense exercise. These results were confirmed by immunoblot accompanied by densitometric analysis.

Key words: Adrenal gland, Training, Morphology, Electron microscopy

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Introduction

Exercise training may be defined as a repetition of exercise bouts over time, which results in enhanced work capacity (Laursen, 2010). This is mainly due to metabolic and systemic adaptations aiming to re-establish and maintain a condition of homeostasis which is disrupted during each acute exercise session. The main adaptations induced by exercise training involve the skeletal muscles. It is well established that regular exercise improves the energy status of the working muscles (Del Balso and Cafarelli, 2007; Laursen, 2010), with a consequent increase in the aerobic capacity (Jones and Carter, 2000; Laursen and Rhodes, 2001) mainly through improved ability in producing and utilizing ATP (Green, 2000), and enhances skeletal muscle oxidative capacity and mitochondrial biogenesis (Moraska et al., 2000).

While improvements in muscle metabolism represent a constant effect of training, a number of different physiological adaptations are also induced in the cardiovascular (Tabata et al., 1990; Schulze et al., 2002; Thompson et al., 2003), immune (Pedersen and Hoffman-Goetz, 2000), and central nervous systems

(Karsten and Geschwind, 2005; Van Praag et al., 2005).

In this respect, it is current opinion that training represents a health-saving factor, which can play an important role in the prevention and treatment of several diseases, as well as preserving intact or even improving some physiological functions. In fact, regular physical activity is associated with decreased heart rate and blood pressure (Kramer et al., 2000; Lee et al., 2010; Gopinath et al., 2011), which in turn lowers the risk of recurrent cardiac events and hypertension (Petroviç-Oggiano et al., 2010; Molmen-Hansen et al., 2011). Moreover, there is evidence that exercise increases hippocampal neurogenesis (Van Praag et al., 1999a,b; Bilang-Bleuel et al., 2000; Olson et al., 2006) associated with enhanced performance in learning tasks (Van Praag et al., 1999a,b), counteracts or delays the loss of cognitive abilities with aging (Larson et al., 2006; Ang et al., 2010), and prevents the occurrence of several psychiatric disorders such as depression (Hagberg et al., 2000; Thompson et al., 2003; Hill and Wyatt, 2005; La Monte et al., 2005; Larun et al., 2006).

However, along with the aforementioned positive effects, typical stress-induced responses are also described after physical exercise (Villaneueva et al., 1986; Luger et al., 1987; Duclos et al., 2001), including a decrease in the activity of the immune system (Moraska et al., 2000; Nieman, 2000; Brown et al., 2007). Evidence suggests that exercise represents a serious risk factor for the human health if played in absence of any adaptation to physical stress (Angeli et al., 2004; Shephard, 2005; Purvis et al., 2010). A number of studies have shown that the negative and potential dangerous effects of physical exercise appear when physical activity is carried out at high intensity, far in excess compared with the individual resistance to stress, as occurs in the "overtraining syndrome" (Chen and Brzyski, 1999; Warren and Stiehl, 1999; Nieman, 2000; Proske and Morgan, 2001; Feasson et al., 2002; Angeli et al., 2004; Shephard, 2005; Purvis et al., 2010).

The adrenal gland plays a central role in the pathophysiology of stress (Selye, 1950; Smith and Vale, 2006) and most stress responses observed after physical exercise appear as the consequence of exercise-induced alteration of the adrenal activity. In fact, it was demonstrated that the adrenal gland represents an early target of physical exercise (Luger at al., 1987; Coleman et al., 1998; Rittenhouse et al., 2002; Brown et al., 2007), resulting in increased plasma concentration of glucocorticoids (Livezey et al., 1985; De Boer et al., 1988; Pagotto et al., 2001), increased adrenal content and release of catecholamines (Scomparin et al., 2006, 2009) and adrenal hypertrophy (Moraska et al., 2000). In these studies acute or repeated physical exercise was mainly considered as a stressful stimulus, and the functional significance of the adrenal-mediated effects of physical exercise was considered as a typical stress response (Katz et al., 1981; Villaneueva et al., 1986; Luger et al., 1987; Ottenweller et al., 1989; Scribner et al., 1993; Fleshner et al., 1995; Coleman et al., 1998; Duclos et al., 2001; Brown et al., 2007).

In contrast, the effects of a specific exercise training on the adrenal gland have been poorly investigated. On the other hand, exercise training, providing a controlled schedule of physical activity which might be modulated during time, should be distinguished from other types of physical exercise. This is substantiated by evidence showing that prolonged and controlled physical activity triggers a pattern of systemic responses which appear to be protective against stress (Tharp, 1975; Sasse et al., 2008).

Since individual resistance to physical stress is critical to the final quality (beneficial or adverse) of the systemic effects of exercise (Petroviç-Oggiano et al., 2010), and training is important in preparing the organism to appropriately sustain physical stress, selecting the most efficient training protocol is determinant in order to produce positive effects.

Therefore, in an attempt to study the effect of distinct training schedules, characterized by different activity patterns, in the present study we investigated in mice the morphological and biochemical effects of two different training protocols on the adrenal gland. More in detail, two different types of exercise training were compared, consisting of: a) brief sequences of intense exercise (corresponding to 90% of maximal intensity) interspersed with recovery periods; and b) continuous exercise at moderate activity (corresponding to 60% of maximal intensity).

Plasma levels of lactate and body weight were measured to assess, respectively, aerobic endurance capacity (Svedahl and MacIntosh, 2003) and general metabolic effects of training in exercising mice compared with control (sedentary) mice (Walberg et al., 1982; Dumke et al., 2001). The effects of training on the adrenal gland were studied at morphological and ultrastructural levels. In addition, immunohistochemistry and immunoblotting analysis were performed to investigate the expression of the catecholamine-synthesizing enzymes within the adrenal medulla.

Materials and methods

Animals

Male C57BL mice 10 weeks old (n=24), weighing 25.8±0.2 g, were used (Harlan Laboratories, San Pietro al Natisone, UD, Italy). One week before starting the experiment, animals were housed four per cage in a temperature-controlled room (20±2°C) with a 12/12 h light/dark cycle and free access to food and water *ad libitum*, except during the experimental sessions (see below). Body weight was recorded at the beginning of training and once a week until the day before the sacrifice. All experimental procedures were performed in accordance with the ethical committee of the University of Pisa and the European Council directive

(86/609/EEC) for the use and care of laboratory animals.

Incremental exercise test

In a preliminary phase of the experimental procedure mice were familiarized with running on a motorized treadmill (Columbus Instrument, Columbus, OH) for 10 min/session at a velocity of 8 $m \cdot min^{-1}$ and 0% grade, twice a day for 1 week. At the end of this period of adaptation, mice underwent an incremental exercise test for five consecutive days (from Monday to Friday). The last trial was performed to establish for each mouse the highest running velocity, then expressed as maximal velocity. To this purpose, mice were initially run at a velocity of 6 $m \cdot min^{-1}$ for 3 min at 0% grade, with the velocity progressively increased by 3 $m \cdot min^{-1}$ every 3 min, until mice were unable to maintain the required running intensity (Ferreira et al., 2007). The maximal velocity was then used to calculate the running velocity for the two different training groups (see paragraph below).

Training

After the incremental test, distance to run was determined, by considering the mean distance covered during 45-60 min of continuous (moderate) running (modified by Ferreira et al., 2007). Such a distance was fixed to 1,000 m, thus keeping constant the total workload in both exercise trainings.

Based on values of maximal running velocity obtained in the incremental exercise test, mice were divided into 3 training groups (n=8 mice for each group), characterized by different activity patterns:

1) High-intensity interval running, consisting of a sequence of short periods of intense effort (2 min running -1 min recovery) at 90% of the maximal running velocity (HIT).

2) Continuous submaximal running at 60% of the maximal running velocity (LOW).

3) Unexercised (sedentary) mice (CON).

Training was performed on a motorized treadmill starting at 10 am once a day (Monday to Friday) for 8 weeks (40 sessions in total). To avoid bias due to different environmental stimulation, at the end of each experimental session sedentary mice were placed for 30 min on the treadmill which had been previously turned off.

One hour before the physical exercise, food was removed from cages. To highlight differences in the body weight increase between groups, mice weight was recorded once a week (Monday).

Blood lactate

Before the 1st and at the end of 1st, 20th and 40th training session, blood lactate concentration was measured. Blood samples were rapidly taken from the tail vein and transferred to 0.2 mL vials containing heparin and were dropped on Bm-Lactate strips (Roche Diagnostics GmbH, Mannheim, Germany). Blood lactate concentration was measured by using an Accutrend/ Accusport Lactate Portable Analyzer (Roche Diagnostics GmbH), according to Bishop (2001).

Tissue preparation, staining procedures and histological analysis

Mice (n=6 per group) were sacrificed by deep anaesthesia using chloral hydrate; for each mouse, one adrenal gland was rapidly dissected out and immersed in Carnoy's fixative solution (60% ethanol absolute, 30% chloroform, 10% acetic acid) for 24h and then transferred in 70% ethylic alcohol overnight at 4°C. Samples were dehydrated in increasing alcohol solutions (80%, 96% and 100% ethylic alcohol), immersed in xylene for 4h, and finally embedded in paraffin. The contralateral gland was assigned to western blot analysis (n=4) or electron microscopy (n=2). Tissue blocks were sectioned using a microtome in order to obtain 7 μm thick slices.

For histological analysis, each sample was completely cut and consecutive serial sections were collected in strict anatomical order on SuperFrost plus™ slides (Cat. No. SUPERFR 1000, Fisher Scientific SAS, Illkirch Cedex - France), dried at 37°C for about 12h, stained with Hematoxylin & Eosin (H&E) and finally coverslipped with DPX plastic mounting media (Cat. No. 44581, Sigma Aldrich, St. Louis, MO USA). Slices were observed at light microscope (Nikon Eclipse 80i, Japan) coupled to a colour videocamera equipped with Nis Element Software (Nikon).

According to Davies et al. (2007), measurement of the cortical and medullary area was performed for each sample by measuring six consecutive adrenal sections corresponding to the inner portion of the adrenal gland. The same sections were also used to measure the relative thickness of the glomerular and fasciculate-reticulate zones of the adrenal cortex. Measurements of the cortical and medullary areas, as well as the extent of the different cortical zones, were obtained using the image analysis software Image J 1.43.

All measurements were carried out at 4x magnification by two different observers, blind to the experimental groups.

Immunohistochemistry

Immunohistochemical analysis was carried out using mouse primary antibody (AbI) against tyrosine-hydroxylase (TH; 1:1000, Cat. No. T1299, Sigma Aldrich), mouse antibody AbI against dopamine-ß-hydroxylase (DBH; 1:500, Cat. No. DBH11-M, Alpha Diagnostic International, San Antonio, Texas USA), rabbit AbI against phenyl-ethanolamine-N-methyl-transferase (PNMT; 1:1000, Cat. No. AB110, Millipore, Billerica, MA USA). Briefly, de-paraffinized and re-hydrated sections were immersed in 0.1% Triton X-100

(Cat. No. T9284, Sigma Aldrich) in TBS for 15 min. Then, sections were quenched for endogenous peroxidase activity (3% H_2O_2 for 10 min), incubated in a blocking solution of 10% normal goat serum (Cat. No. S-1000, Vector Laboratories, Burlingame, CA USA) and TBS, for 1h at room temperature and finally immersed in the above described antibody solutions also containing 2% normal goat serum in TBS overnight at 4°C.

After washing out, slices were incubated with the secondary anti-mouse and anti-rabbit biotinylated antibodies (Cat. No. BA-9200 and BA-1000 respectively,Vector Laboratories,) diluted 1:200 in TBS for 1h, followed by ABC kit (Cat. No. PK-6100, Vector Laboratories) and diaminobenzidine (DAB, Cat. No. SK-4100, Vector Laboratories). Finally, slices were dehydrated in increasing alcohol solutions, mounted with DPX (Sigma Aldrich) and observed at light microscope.

Transmission electron microscopy

Mice (n=2 per group) were anesthetized with intraperitoneal injection of chloral hydrate and then thoracotomized. Subsequently, they were perfused with a fixing solution (2% paraformaldehyde and 0.1% glutaraldeyde in 0.1 M phosphate buffer solution) through the left ventricle. Both adrenal glands of each animal were dissected as described by Tomlinson and Coupland (1990), removed from their pericapsular fat and immersed in the same fixative solution for 1h and 30 min. Samples were then post-fixed in buffered 1% osmium tetroxide for 1h, dehydrated in increasing ethanol solutions and embedded in Epon-araldite. Two additional adrenal glands per group from mice assigned to histological analysis were immersed in the fixative solution (2% paraformaldehyde and 0.1% glutaraldeyde in 0.1 M phosphate buffer solution) for 3h and processed as described above.

Thin sections were cut with ultramicrotome, stained with uranyl acetate and lead citrate and observed with the transmission electron microscope Jeol Jem 100 SX (Japan).

Western Blot assay

Samples of adrenal glands (n=4 per group) were then homogenized in buffer containing 50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS and 10 μg/ml of proteinase inhibitors (leupeptin, pepstatin, aprotinin). Homogenates were centrifuged at 5,000g for 5 min and the supernatant was subsequently centrifuged at 30,000g for 30 min (Rothman et al., 2003). An aliquot of supernatant was used to determine the protein concentration by a protein assay kit (Cat. No. TP0300, Sigma Aldrich). Samples containing 40 Ìg of total protein were solubilized and electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, the proteins were transferred to PVDF membrane (Cat. No. RPN1416F, GE Healthcare, Milan, Italy). The membrane was immersed at 4°C for 3h in blocking solution (5% non fat dried milk in PBS containing 0.05% Tween-20). Subsequently, the membrane was incubated with primary antibody anti-TH (1:1000, Sigma Aldrich), anti-DBH (1:1000, Millipore), or anti-PNMT (1:600, Cat. No. ab90862, Abcam, Cambridge, UK) overnight at 4°C. Blot was probed with horseradish peroxidase-labeled secondary antibody (1:2000 for TH and DBH, 1:1000 for PNMT, Cat. No. RPN2108, GE Healthcare) and the bands were visualized with enhanced chemiluminescence reagents (Cat. No. RPN2108, GE Healthcare). Films of Western blots were scanned and the optical density of the bands was measured using NIH Image 1.61 software. Each result was confirmed in triplicate.

Statistical analysis

Data related to body weight and lactate levels from each mouse were used to obtain the mean value ± SEM for each group. Concerning the morphometrical analysis, the observer's measurements from each mouse were used to obtain the mean values ± SEM of each group.

Finally, values related to the densitometric analysis represent the mean ± SEM of three independent measurements. Comparisons among groups were made by using a one-way ANOVA combined with Scheffè's *posthoc* test. Comparison between blood lactate levels before and after training within the same group was made using Student's t-test for paired data.

Null hypothesis was rejected for $P<0.05$.

Results

Body weight

Table 1 shows the body weight of each experimental group measured before (T_0) and at the end of the training (T_{40}). Body weight measured in CON at T_{40} showed an increase of 12.4±1.4% compared with the weight recorded at T_0, due to the physiological age-related weight increase. The increase in the body weight observed in HIT mice was higher than the increase found in CON mice (16.0±0.5%), suggesting that high-intensity training produces a positive effect on the body

Table 1. Body weight.

	T_0 (gr)	T_{40} (gr)	Increase (%)
CON	25.6±0.5	28.8±0.3	12.4±1.4
LOW	26.0±0.5	28.3±0.6	8.6±0.9*
HIT	25.8±0.3	29.9±0.3#	16.0±0.5*,§

#: $P<0.05$ vs LOW; *: $P<0.05$ vs CON; §: $P<0.001$ vs LOW

growth. In contrast, LOW mice showed a body weight which, at the end of training, was increased significantly less than CON mice (8.6±0.9 %).

Blood lactate

Values related to blood lactate in each experimental group are shown in Table 2. After the first training session (T_1) the blood lactate found in HIT (4.85±0.06) and LOW mice (5.05±0.09) was significantly higher compared with CON (4.30±0.12). At T_{20} blood lactate was similar between groups, whereas at the end of training (T_{40}) lactate concentration in both LOW and HIT mice (3.60±0.12, 2.85±0.12, respectively) became lower compared with blood lactate measured at T_1, as expected by an effective training. In particular, lactate concentrations of HIT mice at T_{40} appeared lower even than those measured in LOW mice (Table 2).

Table 2. Blood lactate.

	T_0 (mmol·L^{-1})	T_1 (mmol·L^{-1})	T_{20} (mmol·L^{-1})	T_{40} (mmol·L^{-1})
CON	4.24±0.08	4.30±0.12	4.33±0.19	4.38±0.10
LOW	4.19±0.11	5.05±0.09*	3.75±0.26	3.60±0.12$^{§,\circ}$
HIT	4.20±0.11	4.85±0.06**	3.95±0.17	2.85±0.12$^{*,\#,\circ}$

*: P<0.0001 vs CON; **: P<0.01 vs CON; §: P<0.001 vs CON; #: P<0.001 vs LOW; °: P<0.0001 vs the same group at T_1

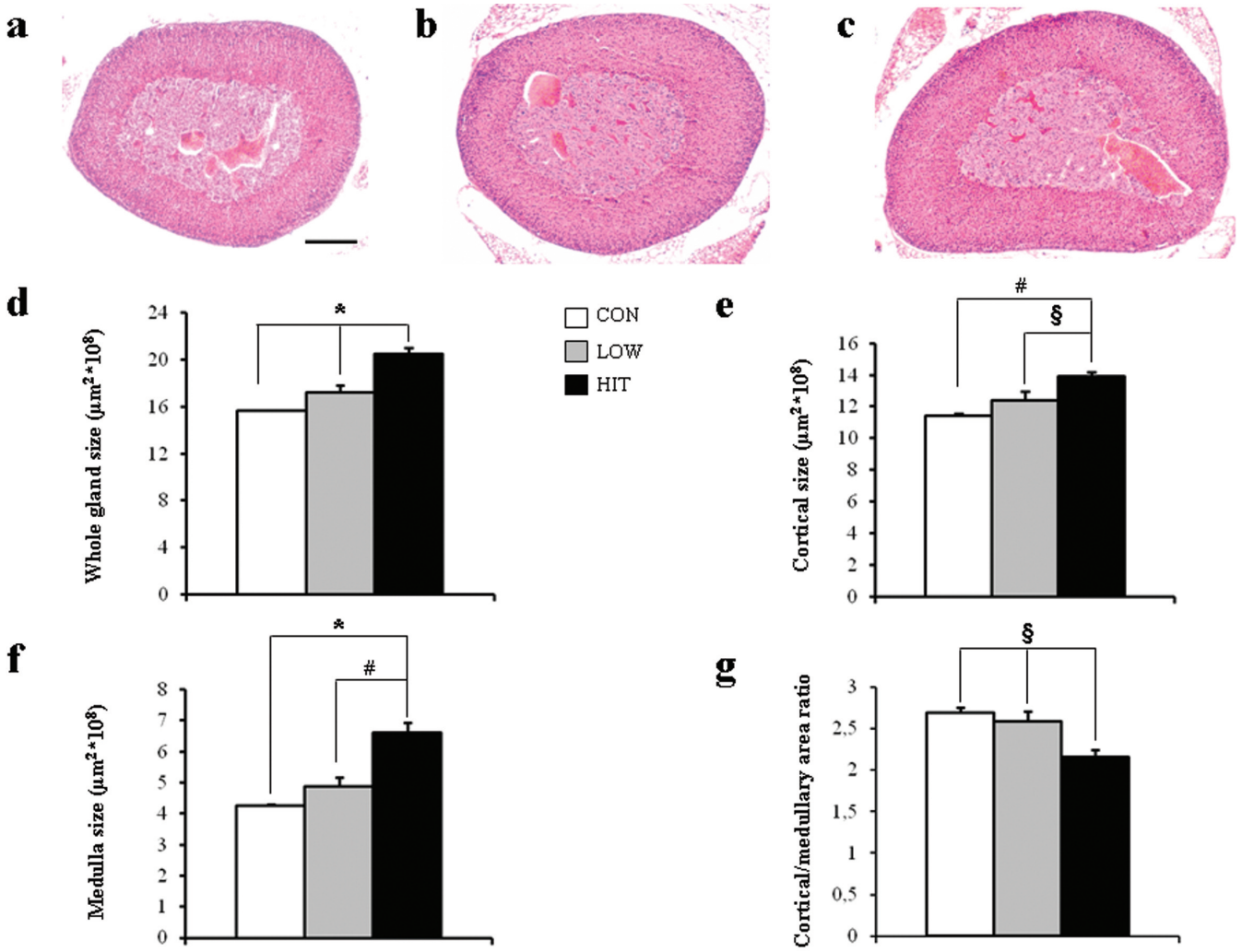

Fig. 1. Morphometric analysis of mouse adrenal gland after exercise training. **a.** Representative H&E-stained section of adrenal gland from untrained control mice (CON), showing a typical medullary portion in the inner part of the section surrounded by the cortex. Exercise training at low **(b)** and high intensity **(c)** produces an increase in the size of the whole gland, which is significant only in HIT mice, as demonstrated by the histograms showing the measurements of the area related to the whole gland **(d)**, the cortex **(e)**, and the medulla **(f)**. Value of the cortex/medulla area ratio is also reported **(g)**. *: P<0.0001; #: P<0.001; §: P<0.05. Scale bar: 270 μm

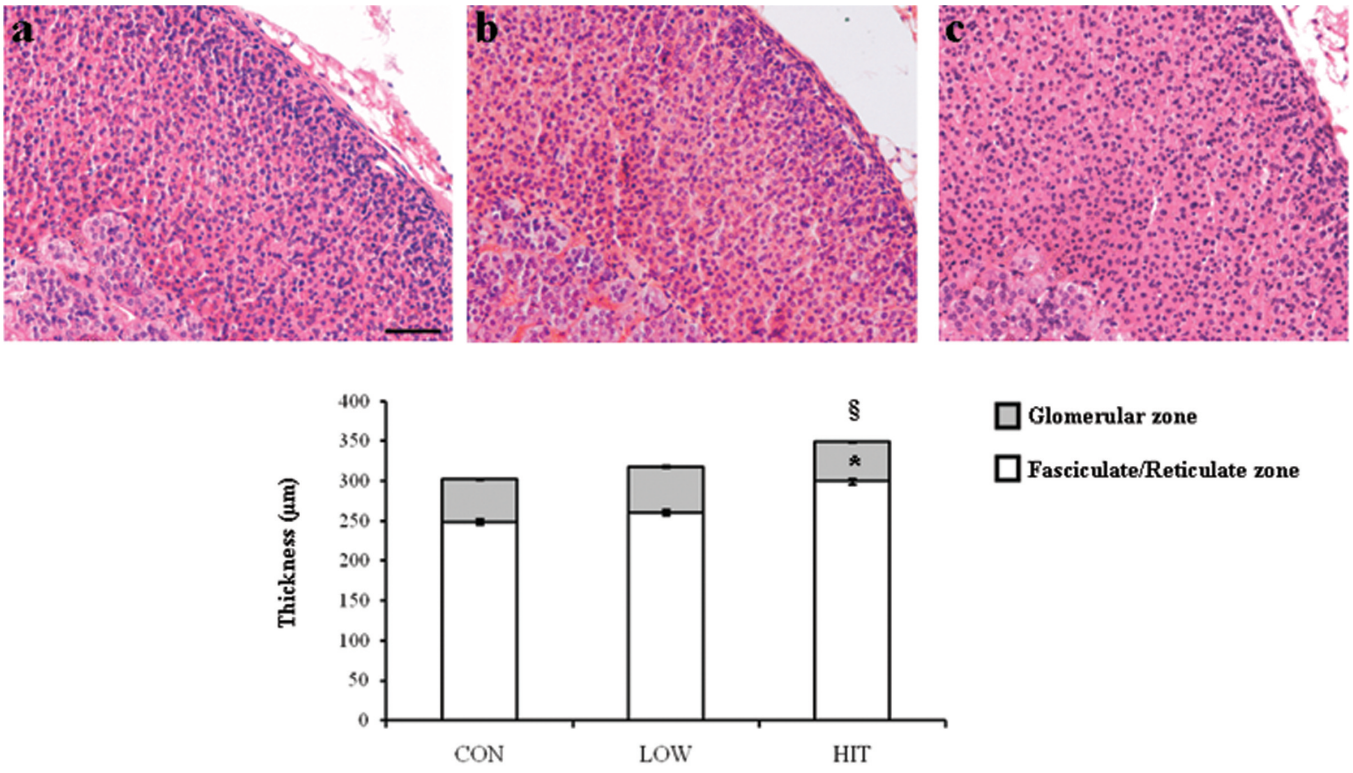

Fig. 2. Morhometric analysis of the mouse adrenal cortex after exercise training. **a.** H&E-stained cortical portion from a CON mouse is shown. Note the typical irregular arrangement of the cell bundles in the outer (glomerular) zone compared with the remaining cortical portion, where they appear radially oriented (fasciculate zone). The inner (reticulate) zone of the cortex is not evident in mice at light microscopy. In HIT mice thickness of the glomerular zone appears reduced compared with CON, whereas the thickness of the fasciculate-reticulate zone increases **(c).** These morphometrical changes are confirmed by the measurement of the relative thickness of the two cortical zones, as shown in the histogram **(d)**. No changes in the relative thickness of glomerular and fasciculate-reticulate zones were found in LOW mice compared with CON (**b, d**). *: $P<0.0001$ vs CON and LOW; §: $P<0.01$ vs CON and LOW. Scale bar: 75 μm

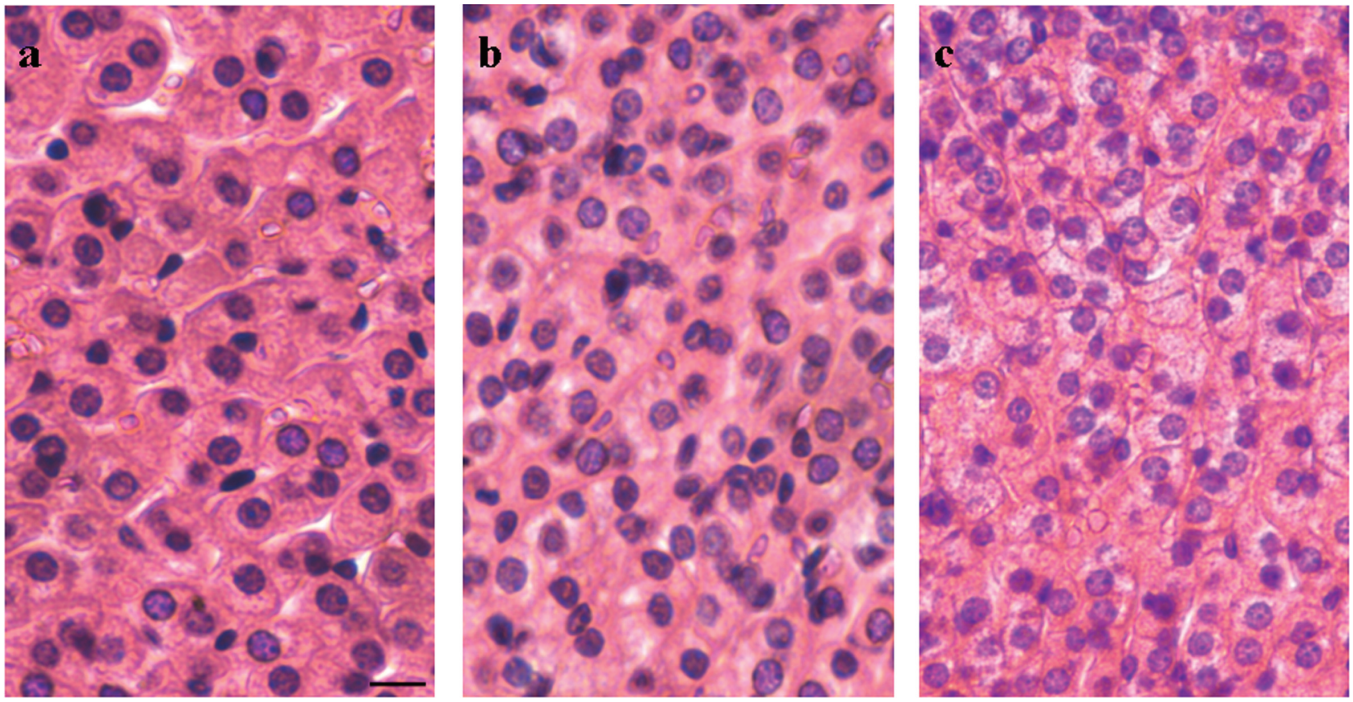

Fig. 3. Morphological changes in the cortical cells of the mouse adrenal gland after exercise training. High magnification of H&E-stained adrenal cortex from a CON mouse **(a)**, and after training, at low- (**b**) or high intensity (**c**). Densely packed cells with diluted cytoplasm appear in both trained mice, but are mainly evident in HIT mice. Scale bar: 12.5 μm

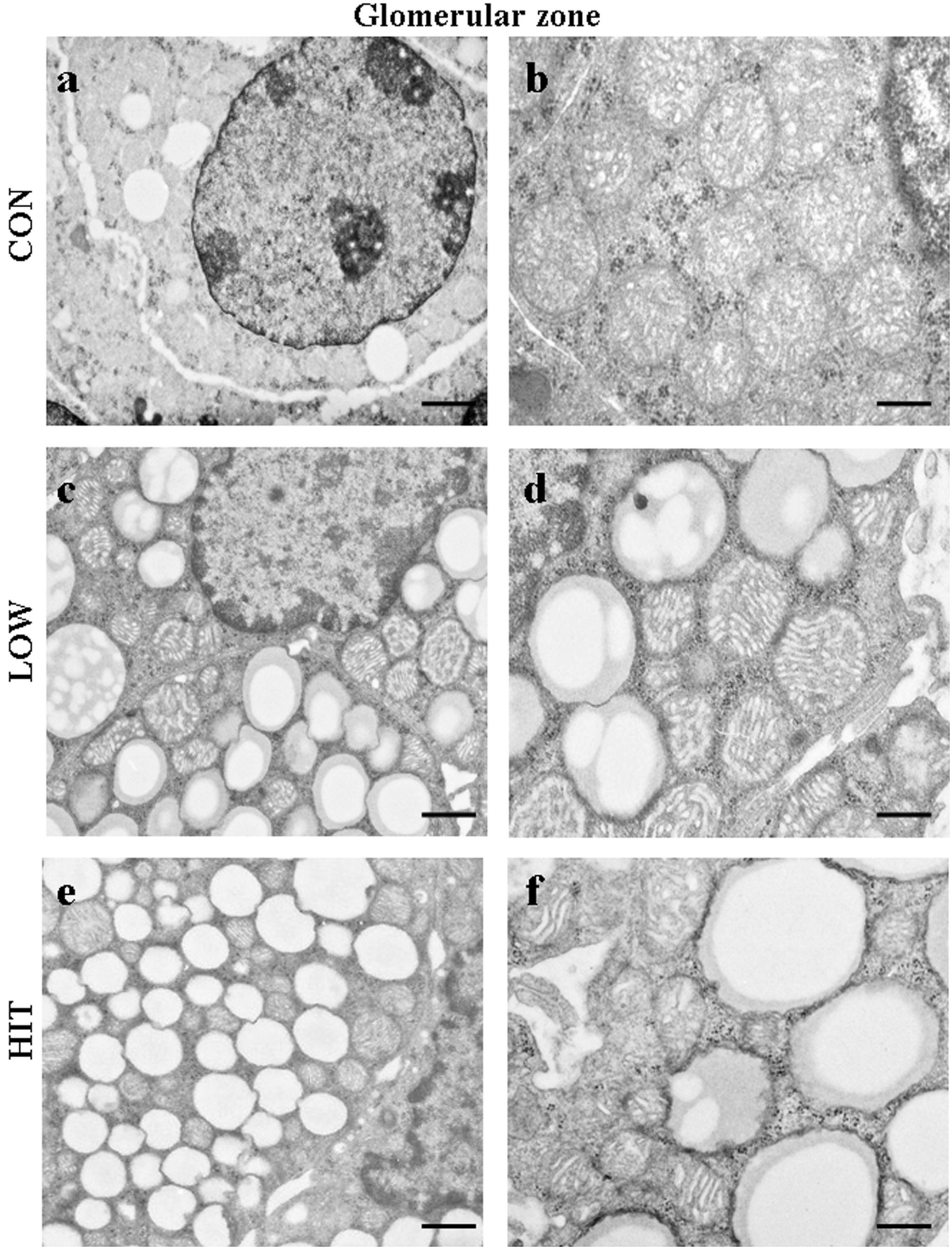

Fig. 4. Ultrastructural effects of exercise training on the glomerular zone in mice. Representative low and high magnification micrographs of glomerular zone cells from CON (**a, b** respectively), LOW (**c, d**) and HIT mice (**e, f**). Note within cells from LOW and HIT mice lipid droplets filling the cytoplasm (**c and e**, respectively) and altered mitochondria with enlarged tubular cristae (**d and f**, respectively). Scale bars: a, 0.8 μm; b, 0.2 μm; c, e, 0.6 μm; d, f, 0.37 μm.

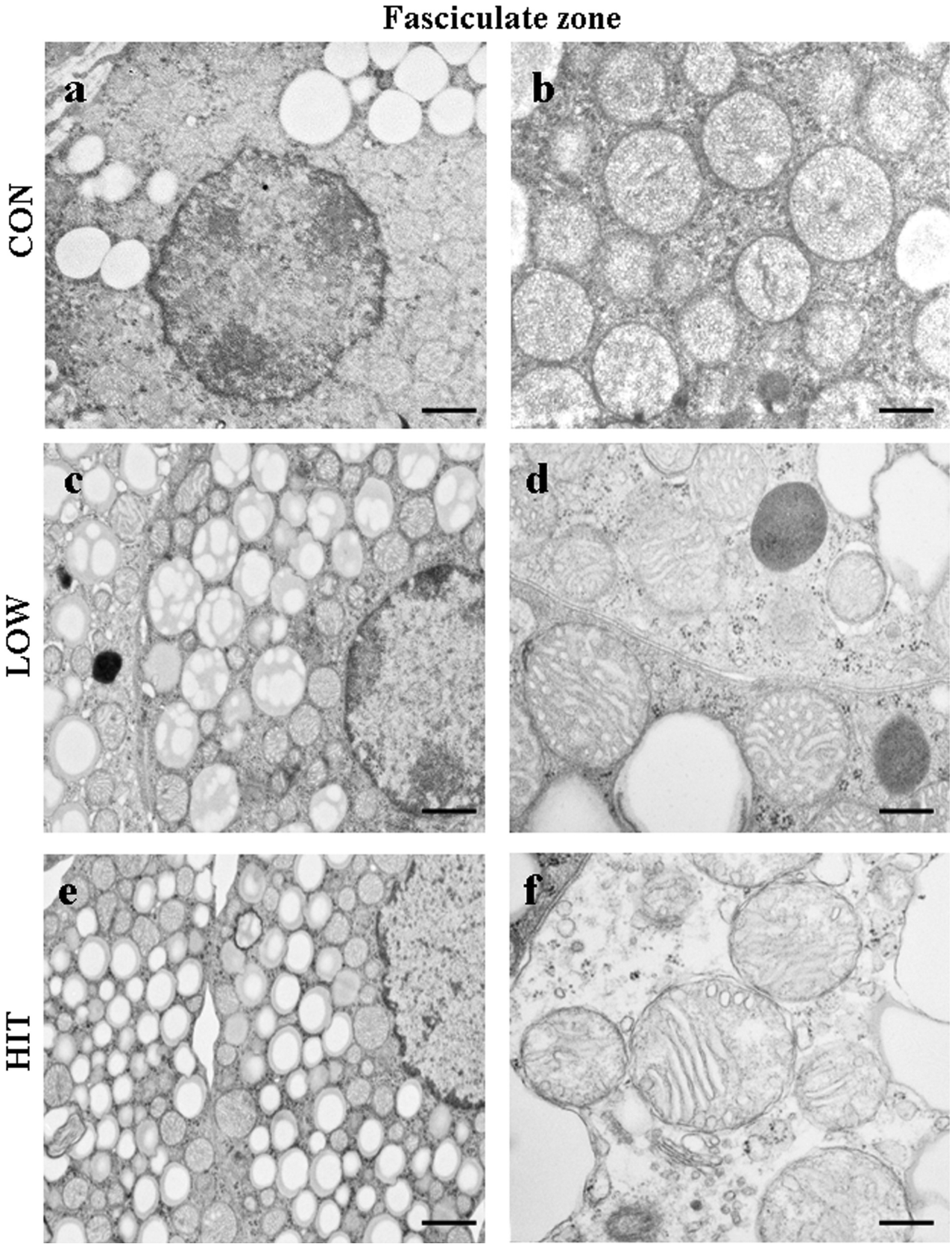

Fig. 5. Ultrastructural effects of exercise training on the fasciculate zone in mice. Low and high magnification TEM micrographs from a CON mouse show cells with well-conformed mitochondria (**a, b** respectively). After exercise training in LOW and HIT mice we observed cells with a large amount of lipid droplets in the cytoplasm (**c and e**, respectively) and mitochondria changes consisting of enlarged tubular cristae in LOW mice (**d)** and severely disarranged cristae and diluted matrix in HIT mice **(f).** Scale bars: a, c, e, 0.7 μm; b, d, f, 0.28 μm.

Morphometric analysis

Morphometric analysis showed that high-intensity exercise training produced a significant increase in the whole gland size (Fig. 1a-c). Measurements of the area related to the inner part of the adrenal gland of HIT mice ($20.532\pm0.497x10^8$ μm^2) revealed an increase of 31.04% and 19.04% compared with CON ($15.668\pm0.133x10^8$ μm^2) and LOW mice ($17.248\pm0.611x10^8$ μm^2), respectively (Fig. 1d). In contrast, low-intensity training did not have any effect on the size of the adrenal gland, which was found unchanged in LOW mice compared

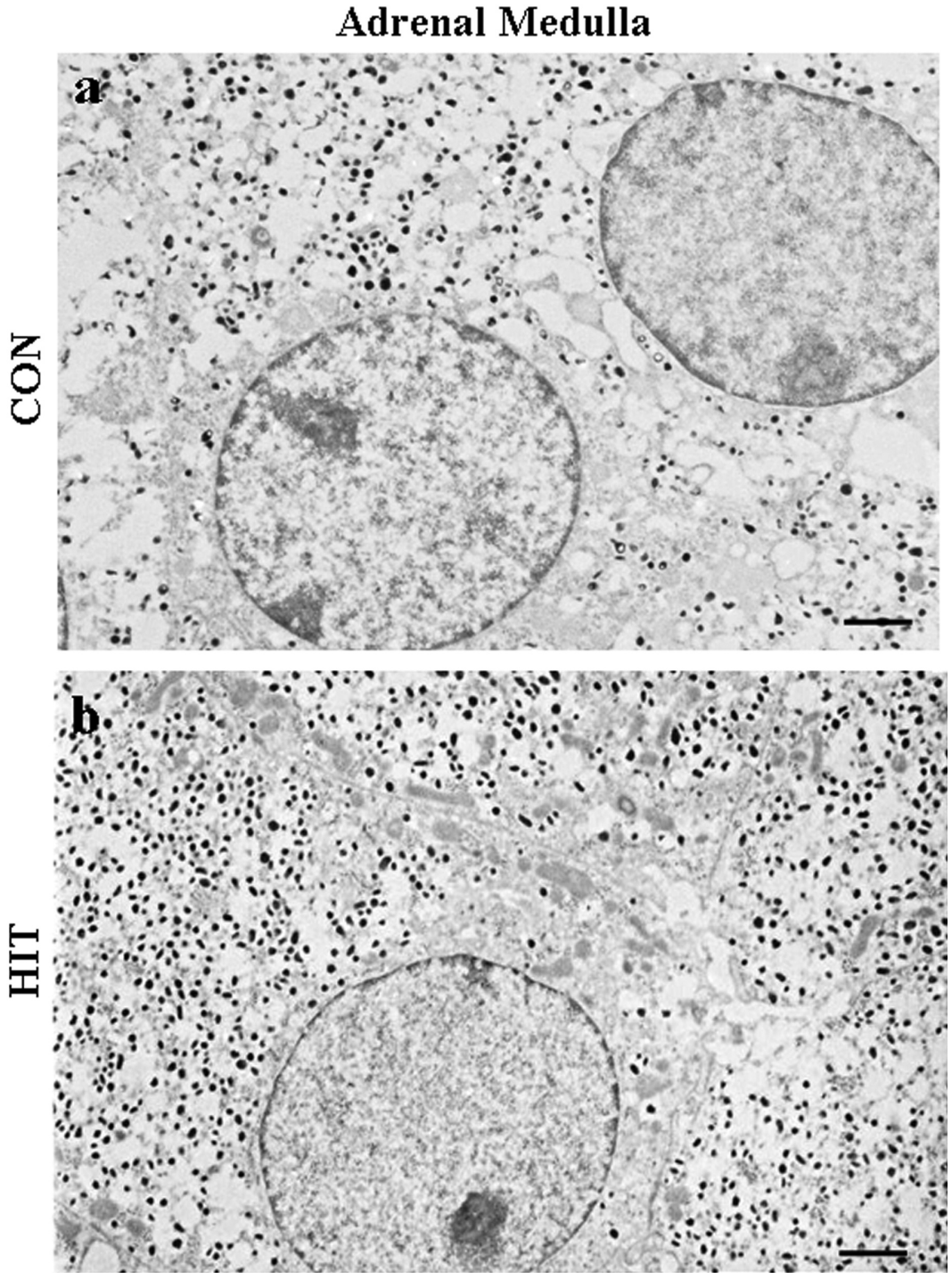

Fig. 6. Ultrastructural effects of exercise training on mouse adrenal medulla. Representative TEM micrograph of adrenal medulla from a CON mouse showing catecholamine cells typically conformed **(a).** After high-intensity exercise training medullary cells appear filled with uniformly distributed catecholamine granules **(b).** Scale bar: 1 μm

with CON (Fig. 1d). The increase in the adrenal gland size in HIT mice involved both cortex and medulla, with a measured area of 13.938±0.242x10^8 μm2 and 6.594±0.340x10^8 μm^2 respectively. In particular, the area of the adrenal cortex measured in HIT mice was 22.06% higher than CON mice (11.419±0.143x10^8 μm^2) and 12.64% higher than LOW mice (12.374±0.613x10^8 μm^2) (Fig. 1e), and even more considerable was the increase found for the adrenal medulla of HIT mice, which appeared 55.19% higher than CON (4.249±0.054x10^8 μm^2) and 35.26% higher than LOW mice (4.875±0.298x10^8 μm^2) (Fig. 1f). These data also indicate that cortex and medulla do not contribute equally to the increase in the whole adrenal gland size found in HIT mice, but this is mainly due to the increase in the size of the adrenal medulla, as confirmed by considering the cortex/medulla area ratio (2.160±0.085, 2.585±0.124 and 2.694±0.056 in HIT, LOW and CON mice, respectively) (Fig. 1g).

In order to better disclose the effect of training in the different cortical zones, we focused our analysis within the adrenal cortex and measured the thickness of the different cortical regions. As shown in Fig. 2a, representing a typical adrenal cortex of a CON mouse, light microscope allows us to clearly distinguish the glomerular and fasciculate zones, on the basis of the morphological features of the cell bundles, whereas it is more difficult to visualize the reticulate zone. Thus, we decided to measure the total thickness of fasciculate and reticulate zone, herewith referred as fasciculate-

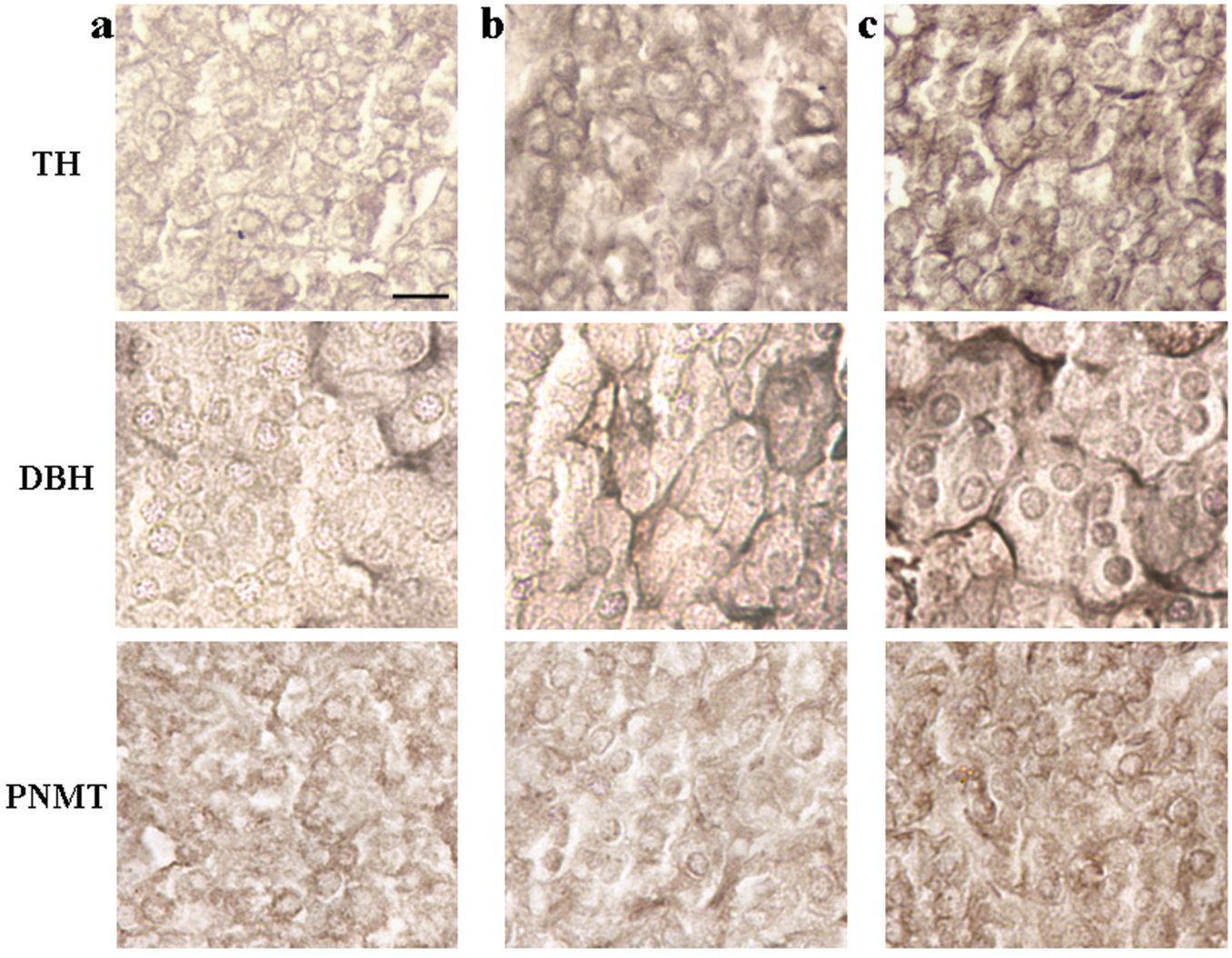

Fig. 7. Immunopositivity for catecholamine-synthesizing enzymes in the mouse adrenal medulla after exercise training. Representative immunoperoxidase for the catecholamine-sinthesizing enzymes tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH), and phenyl-ethanol-amine-N-methyl transferase (PNMT) within the medulla from CON **(a)**, LOW **(b)**, and HIT mice **(c)** are shown. The immunopositivity for all these proteins appears markedly intense in mice after high-intensity exercise training. Scale bar: 20 μm

reticulate zone. We found that high-intensity training produced a significant decrease in the thickness of the glomerular zone in comparison with CON (8.02%) and LOW (13.07%) mice and, at the same time, a significant increase in the extent of the fasciculate-reticulate zone in comparison with CON (20.38%) and LOW (14.84%) mice (Fig. 2). In contrast, no differences in the relative extent of different cortical zones were found in LOW mice compared with CON (Fig. 2d).

Morphological analysis at light and electron microscopy

Analysis at light microscopy of the adrenal cortex of trained mice showed the occurrence of large areas of diluted cytoplasm which were particularly evident in HIT mice (Fig. 3). Moreover, although the adrenal medulla exhibited a more evident size increase after training, any change in the morphological feature was found in the medullary cells when observed at light microscope.

Ultrastructural analysis confirmed these findings and extended more in depth the characterization of the training-induced morphological changes of the adrenal gland. In detail:

Glomerular zone

Cells from CON mice appeared with typical ultrastructural features, characterized by the presence of a few lipid droplets (Fig. 4a), and mitochondria both elongate and round in shape with tubulo-vesicular cristae (Fig. 4b). After low- and high-intensity exercise training the cell cytoplasm appeared filled with lipid droplets (Fig. 4c and 4e, respectively), which were more abundant in HIT mice (Fig. 4e). Moreover, in LOW and HIT mice mitochondrial cristae appeared mainly tubular and enlarged (Fig. 4d and 4f, respectively).

Fasciculate zone

Cells from CON mice showed the typical ultrastructure, with moderately abundant lipid droplets in the cytoplasm and mitochondria exhibiting the typical vesicular cristae (Fig. 5a,b).

After low- and high-intensity exercise training we found cells showing a high amount of lipid droplets filling large areas of the cytoplasm (Fig. 5c and e, respectively). These liposomes were frequently observed in direct contact with mitochondria. In LOW and HIT mice mitochondria cristae appeared mainly tubular and enlarged (Fig. 5d and f, respectively). In particular, high-intensity training appeared to produce a worsening in the mitochondria ultrastructure characterized by disarranged cristae and reduced matrix density (Fig. 5f).

Reticulate zone

Analysis of the reticulate zone showed no ultrastructural differences between groups (data not shown). In particular, after low- and high-intensity training the scarce lipid content and the tubulovesicular mitochondria cristae typically observed in the CON mice

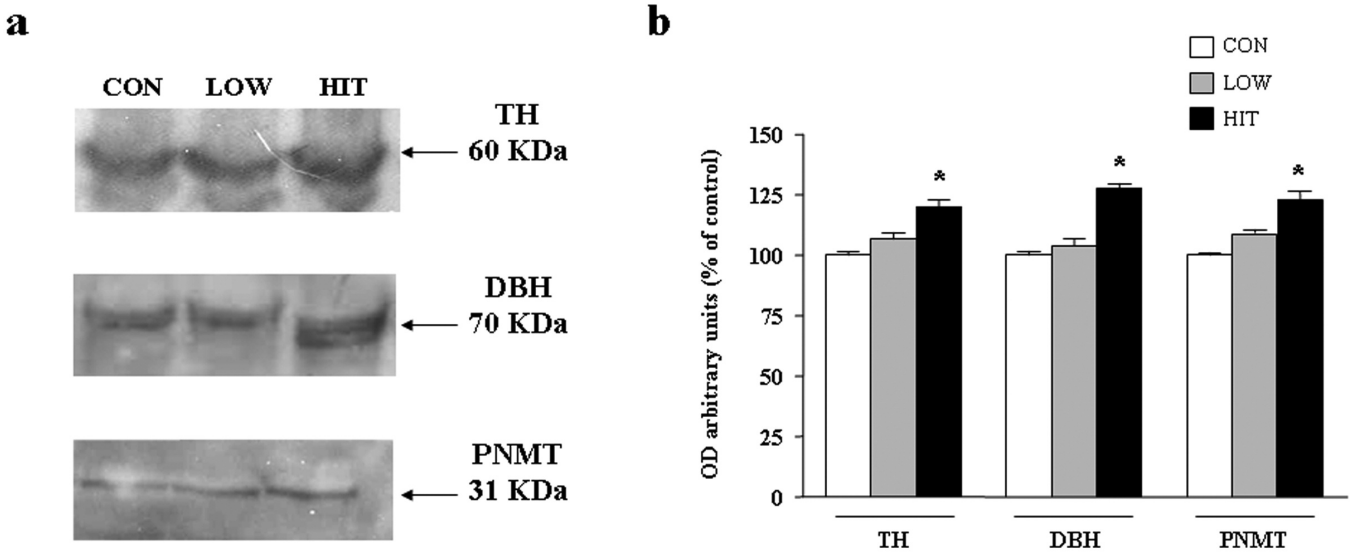

Figure 8. Immunoblotting for catecholamine-synthesizing enzymes in the mouse adrenal medulla after exercise training. **a.** Representative western blotting of tyrosine hydroxylase (TH), dopamine-,-hydroxylase (DBH), and phenyl-ethanol-amine-N-methyl transferase (PNMT) in exercise-trained mice. **b.** Densitometric analysis of the immunoblotting gels is expressed as arbitrary units. Data represent the mean ± SEM of three independent experiments. *P<0.05 vs CON and LOW.

remain unchanged (data not shown).

Adrenal medulla

Typical ultrastructural features of catecholamine-containing cells appeared in the adrenal medulla from CON mice (Fig. 6a). Low-intensity training did not produce any change in medullary cells (data not shown).

In contrast, high-intensity training caused an increase in the catecholamine-containing granules, which appeared homogeneously distributed within the cytoplasm of medullary cells from HIT mice (Fig. 6b).

Analysis of catecholamine-synthesizing enzymes

To investigate the potential effect of training on catecholamine synthesis by adrenal medulla, immunohistochemistry was carried out with antibodies against the enzymes responsible for the synthesis of the catecholamines (i.e., noradrenaline and adrenaline). Representative TH, DBH and PNMT immunostaining in CON, LOW and HIT mice are reported in Fig. 7, which shows the occurrence of an increase in the immunopositivity for all these markers only in HIT mice.

These qualitative results were confirmed by immunoblotting coupled with densitometric analysis, which demonstrated a significant increase in TH, DBH and PNMT protein content in HIT mice compared with CON and LOW mice (Fig. 8).

In line with other data obtained in this experiment, the mild increase in TH, DBH and PNMT immunopositivity observed in LOW mice (Figs. 7, 8a) was not significant compared with CON, as shown by densitometric analysis (Fig. 8b).

Discussion

The present study shows that prolonged high-intensity exercise training produces specific morphological and biochemical changes in adrenal gland of mice, which involves both the adrenal cortex and medulla, where an altered expression of the catecholamine-synthesizing enzymes was also found. These changes are also accompanied by a reduction in blood lactate after exercise and an increase in body weight.

In order to verify the efficacy of training in inducing adaptation to physical effort (Gobatto et al., 2001; Manchado et al., 2005; Philp et al., 2005), measurements of blood lactate were performed at baseline and at different times during exercise training (i.e., 1st, 20th, and 40th training sessions). In both LOW and HIT mice we found that blood lactate was higher than CON after the first session, and it drastically dropped at the end of training, becoming lower than both CON and LOW mice. In particular, at the end of training, blood lactate from HIT mice was significantly lower even than LOW mice. These data indicate a successful adaptation to exercise effort after training.

When considering the variation in the body weight at the end of the training, whereas HIT mice showed a marked increase in body weight, higher than the physiological increase recorded in CON mice, in LOW mice body weight appeared decreased, even compared with CON. This is consistent with the evidence that high-intensity exercise, through a major muscle activation, produces an increase in muscle mass (LeBrasseur et al., 2010; Peterson et al., 2011); in contrast, when exercise occurs as a continuous moderate activity, it is unable to produce any clear effect on muscle mass, but results in an increased basic metabolism, which through an increased lipid consumption may lead to a reduction in body weight (Hansen et al., 2007; Joseph et al., 2011).

Moreover, our study demonstrated that distinct exercise training protocols, characterized by different intensity, cause specific morphological alterations in the mouse adrenal gland. These changes are more evident in HIT mice, whereas mild alterations found in LOW mice appear not to be significant compared with CON.

Morphometric analysis showed that high-intensity training produces an increase in the whole size of the adrenal gland (measured as total area of the section corresponding to the middle portion of the gland) compared with both LOW and CON mice. In particular, our data show that such an increase in the gland size found in HIT mice is mainly due to the increase in the adrenal medulla, which was 55.19% and 35.26% higher than CON and LOW mice, respectively, whereas the size increase in adrenal cortex in HIT mice was 22.06% and 12.64% higher compared with CON and LOW mice, respectively. Moreover, the adrenal cortex of HIT mice showed changes in the relative extent of the different cortical zones, consisting of an increase in the thickness of the fasciculate-reticulate zone (20.38% and 14.84% higher compared with CON and LOW mice, respectively) and a concomitant decrease of the glomerular zone (8.02% and 13.07% lower compared with CON and LOW mice, respectively).

These data are in agreement with previous findings demonstrating that chronic exercise produces adrenal hypertrophy (Tharp, 1975; Moraska et al., 2000) and an increase in the width of the fasciculate zone (Buuck et al., 1976).

Moreover, there is strong evidence suggesting that the increase in the size of the adrenal gland after a stressful stimulation indicates an increase in the functional activity of the gland. This was demonstrated by several studies which showed that following exercise (Tharp, 1975), stress (Akana et al., 1983; Ulrich-Lai et al., 2006) or immunosuppressive drugs (Bryant et al., 1991) an increase in the functional activity of the adrenal cortex leading to increased glucocorticoids levels is constantly accompanied by an increase in the adrenal gland whole size.

To investigate more in depth the effect of training on cell morphology, a careful analysis at light microscope

showed that cortical cells of both HIT and LOW mice exhibited large areas of diluted cytoplasm, which when observed at transmission electron microscope, appeared as lipid vacuoles filling the cytoplasm. These lipid vacuoles were localized both within the glomerular and fasciculate zones, and appeared particularly abundant in HIT mice, which also exhibited peculiar ultrastructural changes within the fasciculate zone, consisting of altered mitochondria with disarranged cristae and reduced matrix density.

An increase in the number of lipid droplets was reported after chronic exercise within the rat adrenal cortex (Buuck et al., 1976). Lipid vacuoles in the adrenal cortex represent deposits where precursors of steroid hormones are stored (Nussdorfer, 1986; Matysiak and Jodłowska-Jędrych 2010).

Our morphometrical data concerning the increase in the thickness of adrenal cortex and fasciculate zone of HIT mice, together with ultrastructural findings which showed training-induced accumulation of lipid droplets within the glomerular and fasciculate zones, may be largely suggestive of an increased activity of the adrenal cortex. However, in the absence of any functional assay our findings *per se* do not allow us to make any conclusive statements about the effective release of cortical hormones. In fact, we cannot rule out that, after chronic exercise, potent induction of the synthesis of cortical hormones may trigger compensatory responses which cause a reduction in hormone release. This might be consistent with low glucocorticoid levels found in the plasma of trained rats after exercise (Tharp, 1975; Sasse et al., 2008; Nyhuis et al., 2010).

Morphological and histochemical changes of cortical adrenal gland were described in different conditions and related to intrinsic enzymatic activity (Greep and Deane, 1947, 1949; Deane and Masson, 1951; Rubin et al., 1963).

It will be worth investigating the real functional significance of the morphological changes we found in our trained mice. In this respect, it will be of interest also to study the significance of mitochondrial alterations occurring within the fasciculate zone of HIT mice. We previously described ultrastructural alterations of mitochondria within the adrenal cortex of rats after noise stress (Pellegrini et al., 1997; Soldani et al., 1999; Gesi et al., 2001), indicating that mitochondria are critically targeted by a variety of stressful stimuli and may reflect stress-induced changes in cellular activity.

In line with this, the fine ultrastructure of mitochondria, which contain enzymes involved in the synthesis of steroid hormones (Privalle et al., 1983; Stocco and Clark, 1996), has been related to the functional activity of the adrenal gland in steroidogenesis (Nussdorfer et al., 1977; Nussdorfer and Mazzocchi 1982; Mazzocchi et al., 1986; Nussdorfer, 1986). Therefore, further studies are needed to clarify the functional significance of the worsening in the ultrastructural features of mitochondria found in the fasciculate zone of HIT mice.

Besides the effects on adrenal cortex, our data show that exercise training also produces changes in the adrenal medulla. Similarly to that described for the cortex, also in the medulla significant changes induced by training appeared only in HIT mice. In these mice an increase in the size of the adrenal medulla was higher than the concomitant increase in the size of the cortex (as documented by the lower cortex/medulla area ratio in HIT mice compared with both LOW and CON mice). However, despite the elevated increase in size of the adrenal medulla, we cannot find any morphological alterations in the catecholamine cells of HIT mice, except for an apparent increase in the catecholamine granules, homogeneously distributed within the cytosol of medullary cells.

By using immunohistochemistry we showed that in HIT mice an increased immunopositivity was found for all catecholamine-synthesizing enzymes (i.e., TH, DBH, and PNMT) and it was further confirmed by western blot analysis followed by immunoblotting for the same primary antibodies. These findings, together with increased accumulation of catecholamine secretory vesicles found at electron microscope within medullary cells of HIT mice, strongly suggest that an enhanced catecholamine synthesis really occurred in these mice.

An increase in enzymes catalyzing catecholamine biosynthesis was described after exposure to stressful stimuli (Sabban and Kvetnansky, 2001), leading to increased levels of catecholamines in the blood stream (McCarty, 1985; Colomer et al., 2009). Increased adrenal catecholamine secretion has also been observed after acute (Bunt, 1986) or chronic (Scomparin et al., 2006) physical exercise. Catecholamines regulate many peripheral functions which are involved in the physiological response to exercise, and their role is prominent in the cardiovascular system, which is markedly involved in sustaining physical effort during exercise training (Perrino et al., 2011). It is well known that catecholamines stimulate cardiac function, producing positive inotropic (increase heart contracting force), chronotropic (increased heart rate), dromotropic (increased conductivity) and bathmotropic effects (increased threshold of excitation) (Opie, 1998; Currie, 2010). Moreover, following stressful stimulation, including physical exercise, adrenaline is able to modify peripheral vascular resistance inducing a powerful vasodilatation within the selected area, which specifically involves skeletal muscles (Vandeputte et al., 2003; Shafaroudi et al., 2005). This peculiar effect of adrenaline is mediated by its action on specific receptors and results in an increased supply of oxygen and nutrients for the actively contracting muscles (Halliwill, 2003).

It is of note that increased expression of the rate-limiting enzyme TH is sufficient by itself to enhance catecholamine biosynthesis. Interestingly, our findings indicate that the expression of the downstream enzymes is also increased in HIT mice, suggesting that this specific training is able to trigger other mechanisms

which, through the increase in the expression of PNMT, lead to an increased adrenaline synthesis by adrenal medulla. In fact, glucocorticoids secreted by the fasciculate zone stimulate the synthesis of PNMT within the adrenal medulla (Jiang et al., 1989; Tai et al., 2007), thus promoting conversion of noradrenaline to adrenaline (Wurtman and Axelrod, 1965). This occurs because of a peculiar vascular dispositive, which allows vessels which have supplied cortical cells to undergo a second capillarization within the adrenal medulla, thus enabling cortical deriving hormones (i.e. glucocorticoids) to produce fast effects on the medullary cells (Wurtman, 2002). This might explain the simultaneous increase of glucocorticoids and adrenaline induced by stress (Axelrod and Reisine, 1984; Chrousos and Gold, 1992), and the attenuated PNMT activity after stress shown in rodents with an impaired endogenous glucocorticoid synthesis (Betito et al., 1994).

The lack of any morphological alteration in LOW mice does not exclude the occurrence of compensatory changes in several functional parameters in these mice. Data referring to blood lactate and body weight, which decrease at the end of training in LOW mice, suggest that in these mice low-intensity training was able to establish a peculiar morpho-functional homeostasis, which leaves the morphological features of the adrenal gland unchanged.

In this respect, since the activity patterns of many sports consist of combining brief periods of high intense exercise with longer periods of moderate- and low-(submaximal) intensity activity (or rest) (Glaister, 2005; Spencer et al., 2005; Ben Abdelkrim et al., 2007; Sheppard et al., 2007; Buchheit et al., 2010) our results might be predictive of potential morphological alterations which may occur in athletes following long periods of sport activity. Further investigations are needed to clarify the functional significance of these training-induced adrenal gland alterations.

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