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Low-Intensity Aerobic Exercise Mitigates Exercise-Induced Bronchoconstriction by Improving the Function of Adrenal Medullary Chromaffin Cells in Asthmatic Rats

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Exercise is one of the most common triggers of bronchoconstriction in patients with asthma. The low levels of circulating epinephrine produced by the adrenal medullary chromaffin cells (AMCCs) are associated with exercise-induced bronchoconstriction (EIB) in asthmatics. In the present study, we tested the hypothesis that low-intensity aerobic exercise may ameliorate EIB using a rat model of asthma. Male Sprague-Dawley rats at 7 weeks of age, sensitized with ovalbumin or treated with saline, were subjected to low or moderate exercise training (50 or 75% of maximum velocity) for one hour in a treadmill 30 min after ovalbumin or saline inhalation. The exercise capacity, airway responsiveness, lung morphology, the morphological changes and endocrine function of AMCCs were measured in both groups of rats after exercise training for 6 weeks. Either low-intensity or moderate-intensity exercise mitigated EIB and increased exercise capacity in ovalbumin-sensitized (asthmatic) rats. Low-intensity aerobic exercise reduced the vacuolar degeneration degrees, lipid contents, neuronal peripherin and neurofilament-68 expression, demolished neurites, but increased the chromaffin granule density, endocrine chromogranin A and phenylethanolamine N-methyltransferase expression in the adrenal medullary tissues, accompanied by increased levels of circulating epinephrine and corticosterone, but decreased nerve growth factor in asthmatic rats. Finally, low-intensity aerobic exercise significantly reduced the relative levels of phosphorylated extracellular signal-regulated kinase and phosphorylated cAMP responsive element-binding protein and the relative mRNA expression levels of downstream molecules, including c-FOS and c-JUN in the adrenal medullary of asthmatic rats. We suggest that low-intensity aerobic exercise improves the endocrine dysfunction of AMCCs and mitigates EIB.

Keywords: adrenal medulla; aerobic exercise; asthma; epinephrine; exercise-induced bronchoconstriction
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Introduction

Exercise is one of the most common triggers of bronchoconstriction in patients with asthma. Though exercise-induced bronchoconstriction (EIB) is usually observed among endurance athletes, it is estimated that 60-90% of asthmatic patients experience EIB (Dryden et al. 2010). A previous study has suggested that recent asthmatic attack and previous allergic rhinitis appear to be risk factors for EIB (Bransford et al. 1991). Recent studies suggest that regular aerobic exercise can actually improve exercise capacity, bronchial hyperresponsiveness, EIB and lung function, or at least improve the management of asthmatic symptoms in asthmatic patients without causing asthmatic deterioration (Farid et al. 2005; Mendes et al. 2010; Boyd et al. 2012; Avallone and McLeish 2013; Eichenberger et al. 2013). However, little is known about the mechanisms underlying the benefits of regular aerobic exercise to asthmatic patients.

Because asthma is an airway inflammatory disorder, previous studies about the effect of aerobic exercise on asthma mainly concentrate on airway inflammation and remodeling. It is well known that low- or moderate-intensity aerobic exercise mitigates airway inflammation and remodeling in a mouse model of asthma (Vieira et al. 2007). Epinephrine is produced by the adrenal medulla and can dilate bronchia. Phenylethanolamine N-methyltransferase (PNMT) is primarily expressed in the adrenal medulla (Kvetnansky et al. 2006) and is the rate-limiting enzyme that catalyzes the conversion of norepinephrine to epinephrine. The activity of PNMT is regulated by corticosterone (Wan and Livett 1989), the hypothalamic-pituitary-adrenal

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axis and the sympathoadrenal system (Lemaire et al. 1993; Stachowiak et al. 1988). Interestingly, the concentrations of plasma epinephrine do not increase during the stress of an acute attack of asthma (Ind et al. 1985). In the absence of adrenergic nerves innervating, circulating epinephrine through the adrenergic receptors is responsible for regulating the relaxation of human airway smooth muscles. Hence, the lower levels of circulating epinephrine are impotent to relieve the bronchospasm during an asthmatic attack. Furthermore, exercise challenge greatly elevates the levels of plasma epinephrine in the EIB-free asthmatic children, but only slightly increases the levels of plasma epinephrine in asthmatic children with EIB (Tsuda et al. 1993). Indeed, the degrees of EIB were inversely associated with the percent of net increase in the concentrations of plasma epinephrine in asthmatic children (Kubota et al. 2000). Collectively, these data suggest that sympathoadrenal hypofunction may contribute to the pathophysiology of EIB. A previous study has shown that moderate-intensity aerobic exercise increases the levels of circulating epinephrine and decreases total lung resistance by 60%, which is attenuated by a β-receptor antagonist in asthmatic mice (Hewitt et al. 2010). Hence, the increased levels of circulating epinephrine by moderate-intensity aerobic exercise may be one of the mechanisms underlying the improvement of airway hyperresponsiveness. However, the mechanisms by which aerobic exercise enhances the release of epinephrine have not been clarified.

Our previous studies have shown that adrenal medullary chromaffin cells (AMCCs) have a tendency to transform into neurons, which results in impaired endocrine function of the adrenal medulla, thereby leading to a decrease in the levels of circulating epinephrine in a rat model of asthma (Feng and Hu 2005; Feng et al. 2012; Hu et al. 2012a). In addition, we found that high-intensity exercise not only promoted inflammatory responses and increased the airway resistance, but also induced neuronal transdifferentiation of AMCCs and subsequently decreased the levels of circulating epinephrine while moderate-intensity of exercise reversed these changes (He et al. 2013). It is well known that nerve growth factor (NGF) is a potent inducer of neuronal differentiation of AMCCs while corticosterone stimulates the cells toward a neuroendocrine phenotype, playing an opposite role in regulating the transdifferentiation of AMCCs (Unsicker et al. 1978, 1980; Tischler et al. 1993). However, our previous studies and those of others did not standardize exercise challenge and measure airway responsiveness to exercise, which might not well mimic the occurrence of EIB. More importantly, whether low-intensity aerobic exercise can improve pathologically morphological changes and endocrine dysfunction in AMCCs and ultimately relieve EIB as well as modulate plasma NGF and corticosterone in asthmatic individuals has not been clarified. Moreover, individual asthmatic patients have varying exercise threshold for inducing bronchospasm. Some asthmatic patients with moderate-intensity aerobic exercise may develop EIB. Accordingly, determination of the effect of low-intensity aerobic exercise on pathologically morphological changes and endocrine dysfunction in AMCCs and EIB will be of great significance in management of asthmatic patients in the clinic.

In this study, we employed a rat model of ovalbumin (OVA)-sensitized and -challenged asthma to test the impact of low- or moderate-intensity aerobic exercise on EIB and the structure and function of the adrenal medulla, as well as to investigate the potential mechanisms underlying their beneficial effect. We found that similar to that of moderate-intensity, low-intensity aerobic exercise reduced EIB by improving pathologically morphological changes and endocrine dysfunction in AMCCs and elevating the levels of circulating epinephrine in asthmatic rats.

Materials and Methods

All animal procedures and experimental protocols were established, according to the guidelines of National Institutes of Health and approved by Ethics Committee of Xiangya Hospital, Central South University.

Animals and groups

Male Sprague-Dawley rats at 6 weeks of age and 180-200 g body weight were obtained from the Department of Laboratory Animal Science, Central South University (Changsha, China) and housed in a specific pathogen free facility with standardized control of temperature and humidity and free access to food and water. After being quarantined for one week, the rats were randomized into six groups (n = 8 each): the control group without OVA sensitization/challenge and exercise; the low group of rats with low-intensity aerobic exercise without OVA sensitization/challenge; the moderate group of rats with moderate-intensity aerobic exercise without OVA sensitization/challenge; the OVA group of rats with OVA sensitization/challenge, but without exercise; the OVA + Low group of rats with OVA sensitization/challenge and low-intensity aerobic exercise; and the OVA + Mod group of rats with OVA sensitization/challenge and moderate-intensity aerobic exercise.

OVA sensitization

The rats in the OVA, OVA + Low, and OVA + Mod groups were sensitized intraperitoneally (i.p.) with a mixture of 100 mg OVA (grade V), 100 mg aluminum hydroxide (Sigma-Aldrich, St. Louis, USA), and 6×10^9 heat-killed *Bordetella pertussis* (Beijing Tiantan Biological Products, Beijing, China) in sterile saline (1 ml) on days 0 and 7 (Hu et al. 2012b). The rats in other groups were injected i.p. with saline alone. Beginning on day 14, the OVA-sensitized rats were exposed to increasing concentrations (from 1% to 11%, wt/vol) of aerosolized OVA (30 min/day for five consecutive days/week) for 6 weeks to avoid OVA tolerance (Prado et al. 2005). The rats in other groups were exposed to aerosolized sterile saline only. The experimental procedures are illustrated in Fig. 1.

Maximal running test and exercise training

Maximal running test and exercise protocol were performed on day 11, as described previously (Vieira et al. 2007; Olivo et al. 2012; He et al. 2013). All rats were initially adapted to the treadmill running (ZH-PT, Zhenghua, Huaibei, China) at 10 m/min with 25%

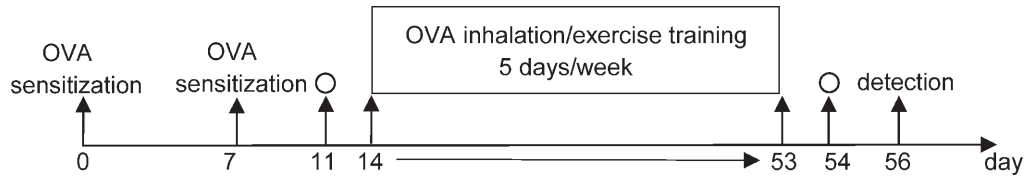

Fig. 1. The time course of the experimental protocol.
Rats were injected with saline or sensitized with OVA in alum on days 0 and 7, and challenged with aerosol saline or OVA five times per week from days 14 to 53. Some rats were subjected to low- or moderate-intensity aerobic exercise training 30 min after the aerosol challenge five times per week. A maximal running test was performed on days 11 and 54 (open circles). The animals were sacrificed and studied on day 56.

incline for 15 min for three consecutive days. A maximal exercise treadmill test was performed to calculate the running velocity for the different training groups of rats. The maximal exercise treadmill test consisted of a 5 min warm-up (25% incline, 10 m/min) and then an increase in treadmill speed (3 m/min every 3 min) until animal exhaustion, a situation in which individual animals were unable to run even after 10 mechanical stimuli. The test was repeated on day 54 (following training for 6 weeks). The exercised rats were trained with 50 or 75% of maximum velocity as the low- or moderate- intensity aerobic exercise 30 min after OVA or saline inhalation beginning on day 14 (60 min/day, 5 days/week) for 6 weeks, respectively.

Exercise challenge and measurement of airway responsiveness

On day 56, rats were challenged with a single bout of 8-min progressive exercise (Kodesh et al. 2011). During the first 4 min, the rats ran on a treadmill with the speed rapidly increasing to 30 m/min and continually running at that speed, until completion of the challenge. This level of exercise was close to the maximal running capability for OVA-sensitized/challenged rats. Before and immediately after exercise challenge, individual rats were subjected to pulmonary auscultation by an experienced observer using a stethoscope (3M™ Littmann®, Allheart, Louisiana, USA) in a blinded manner. Rats were examined three times both before and after exercise challenge (over a 10-min period), and each examination lasted for 1 min. The auscultated sounds were semi-quantitatively classified, as previously described (Kodesh et al. 2011): 0, clear breath sounds with no abnormality; 1, increased breath sounds without elements of tone or pitch; and 2, increased breath sounds with elements of tone or pitch. Following the pulmonary auscultation, the rats were anesthetized by i.p. injection with 10% chloral hydrate solution (3.5 ml/kg). Subsequently, the rats were measured for their airway responsiveness using whole-body plethysmography (PLY 3211, Buxco Electronics, Troy, New York, USA). Increasing doses of aerosolized methacholine (0-20 mg/ml, Sigma Chemical, St. Louis, MO, USA) were delivered, and the airway resistance (RL) of individual rats was calculated by dividing the driving pressure by the rate of air flow (P/V). After measuring airway responsiveness, blood samples were collected by cardiac puncture, and the rats were sacrificed.

Bronchoalveolar lavage

The bronchoalveolar lavage (BAL) was recovered as previously described (Feng et al. 2010). After blood sampling, the right mainstem bronchus was occluded with a clamp and the left lung was lavaged three times via a tracheal cannula with saline (3 ml each time) using a syringe. The recovery of BAL fluid (BALF) was > 70%. The total cell numbers in the BALF samples was estimated using a haemocytometer. The BALF samples were centrifuged (4°C, 1,000 r/min, 10 minutes), the sediments were stained with May-Grunwald-Giemsa and differential types of cells were counted on a total of 200 cells.

Histology

The right middle lung lobe and adrenal glands of individual rats were fixed in 4% paraformaldehyde at 4°C overnight and paraffin-embedded. The lung and adrenal gland tissue sections (4 μm) were stained with hematoxylin and eosin (H & E).

Transmission electron microscopy

The adrenal gland tissue samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 3 hrs, and further fixed in buffered 1% osmium tetroxide (OsO4) for 1 h. The specimens were dehydrated in ethanol and embedded in Epon-Araldite. The ultrathin tissue sections (50 nm) were stained with uranyl acetate and lead citrate, and finally examined under a H-7,500 transmission electron microscope (Hitachi, Tokyo, Japan). The densities of chromaffin granules per gland (/mm^3) were assessed for every rat (Hu et al. 2012a). The numbers of neurite-bearing cells and AMCCs in the adrenal medulla of individual rats were counted and the percentages of neurite-bearing cells in the total numbers of cells were calculated, based on at least thirty cells from two sections of each sample and 4-5 rats per group. All samples were assessed by two independent pathologists in a blinded manner.

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The levels of serum epinephrine, NGF and corticosterone in individual rats were measured using ELISA kits (Abnova, Taipei, Taiwan; Uscn, Wuhan, China; Cayman, Ann Arbor, USA), respectively. The limitation of detection for epinephrine, NGF and corticosterone was 10 pg/ml, 17 pg/ml and 30 pg/ml, respectively.

Western blot analysis

The adrenal medulla was isolated as previously described (Liu et al. 2005; Nostramo et al. 2012). Briefly, the adrenal glands were dissected and frozen on dry ice. A small incision was inserted to the edge of the cortex and the medulla was gently squeezed out. Subsequently, any cortex tissue adhering to the adrenal medulla was carefully removed. It was estimated that the purity of isolated medulla was greater than 90%. The collected adrenal medullary tissues from individual rats were homogenized. After centrifugation and quantification, the protein lysates (30 μg/lane) were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA). The membranes were treated with 0.05 g/ml of skim milk powder at room temperature (20°C) for 2 hrs and

Table 1. Primer sequences.

Primer		Primer sequences	Product size
c-FOS	Forward primer	5′-GGAATTAACCTGGTGCTGGA-3′	200 bp
	Reverse primer	5′-TGAACATGGACGCTGAAGAG-3′	
EGR1	Forward primer	5′-GAGAAAGTTTGCCAGGAGTGAT-3′	227 bp
	Reverse primer	5′-CAGGAGACGGGTAGGTAGAGG-3′	
c-JUN	Forward primer	5′-GAAGTAGCCCCCAACCTCTC-3′	291 bp
	Reverse primer	5′-ATGGCTCTCAACTCAAGCGT-3′	
FOSB	Forward primer	5′-GTGAGAGATTTGCCAGGGTC-3′	129 bp
	Reverse primer	5′-AGAGAGAAGCCGTCAGGTTG-3′	
JUNB	Forward primer	5′-CGCATCAAAGTGGAGCGAAAG-3′	271 bp
	Reverse primer	5′-GGTGTCCGTATGGAGCAAGG-3′	
β-actin	Forward primer	5′-AGGCCCCTCTGAACCCTAAG-3′	202 bp
	Reverse primer	5′-CCAGAGGCATACAGGGACAAC-3′	

incubated with anti-peripherin (1:20,000), anti-neurofilament-68 (NF-68, 1:5,000), anti-chromogranin A (CgA, 1:10,000), anti-PNMT (1:1,000), anti-ERK1/2 (extracellular signal-regulated kinase, 1:1,000), anti-phosphorylated ERK1/2 (anti-p-ERK1/2, 1:500, Abcam, Cambridge, UK), anti-CREB (cAMP responsive element binding protein, 1:1,000), anti-phosphorylated CREB (anti-p-CREB, 1:1,000), or anti-β-actin (1:1,000, Cell Signaling Technology, Beverly, USA) overnight at 4°C. The bound antibodies were detected with horseradish-peroxidase (HRP)-conjugated secondary antibodies and visualized using enhanced chemiluminescence (Beyotime, Haimen, China) detection. The relative levels of target protein to control β-actin or phosphorylated to total protein were determined using the Image Pro Plus 6.0 software (IPP6.0, Media Cybernetics, Rockville, USA).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) measurement

Total RNA was extracted from the adrenal medullary tissues of individual rats using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 1.0 μg total RNA was reversely transcribed into cDNA using a cDNA first-strand synthesis kit (Fermentas Lithuania, Pittsburgh, USA). The relative levels of target gene mRNAs to the control β-actin were determined by quantitative real time PCR using specific primers and SYBR Green PCR Kit in a 7900HR Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The sequences of primers are shown in Table 1. The PCR amplification was performed at 95°C for 10 s and subjected to 35 cycles of 95°C for 10 s, 58°C for 30 s and 60°C for 15 s for the early growth response gene 1 (EGR1), proto oncogene c-Jun (c-JUN) and FBJ murine osteosarcoma viral oncogene homolog B (FOSB) and 95°C for 10 s, 60°C for 45 s for the c-Fos oncogene (c-FOS), JunB proto-oncogene (JUNB) and β-actin. The relative levels of each target gene mRNAs to the control β-actin were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

Data were expressed as the mean ± standard deviation (S.D.). Statistical analysis of data was performed with Statistical Package for the Social Sciences (SPSS) 19.0 software (SPSS, Chicago, USA). Comparisons among groups were performed by one-way analysis of variance (ANOVA) and post hoc Student-Newman-Keuls test. A P value of < 0.05 was considered statistically significant.

Results

Low- or moderate-intensity aerobic exercise mitigates EIB and airway inflammation, and increases exercise capacity in a rat model of asthma

The effects of exercise training on maximal exercise capacity, pulmonary auscultation, airway resistance, and inflammation in the different groups of rats were measured. As shown in Fig. 2A, the periods of treadmill tests in the Low and Mod groups of rats were significantly longer than that in the control group of rats ($P < 0.05$). The periods of treadmill tests in the OVA + Low and OVA + Mod were similar to that in the control group, which was significantly longer than that in the OVA group ($P < 0.05$ for all).

As expected, the healthy rats showed no change in auscultation breath sounds in response to exercise challenge. After exercise, the scores of auscultated sounds increased in the OVA and OVA + Low groups ($P < 0.05$), but not in the OVA + Mod group ($P > 0.05$). Furthermore, low or moderate training significantly reduced the scores of auscultated sounds following exercise challenge in asthmatic rats ($P < 0.05$, Table 2). Similarly, while there was no significant difference in the airway resistance among the different groups of healthy rats, the airway resistance in all asthmatic groups of rats were significantly higher than that in the healthy controls when the concentration of methacholine reached 10 mg/mL and above ($P < 0.05$). Low or moderate training attenuated the airway resistance in asthmatic rats ($P < 0.05$). The airway resistance in the OVA + Mod group tended to be lower than that in the OVA + Low group of rats, but there was not a statistically significant difference (Fig. 2B).

In comparison with that in the healthy rats, the numbers of total cells eosinophils and neutrophils in BALF significantly increased in asthmatic groups ($P < 0.05$). As compared with that in the OVA group, low- and moderate-intensity aerobic exercises decreased the numbers of total cells eosinophils and neutrophils in BALF ($P < 0.05$, Fig. 2C). Histological examination revealed that there was no

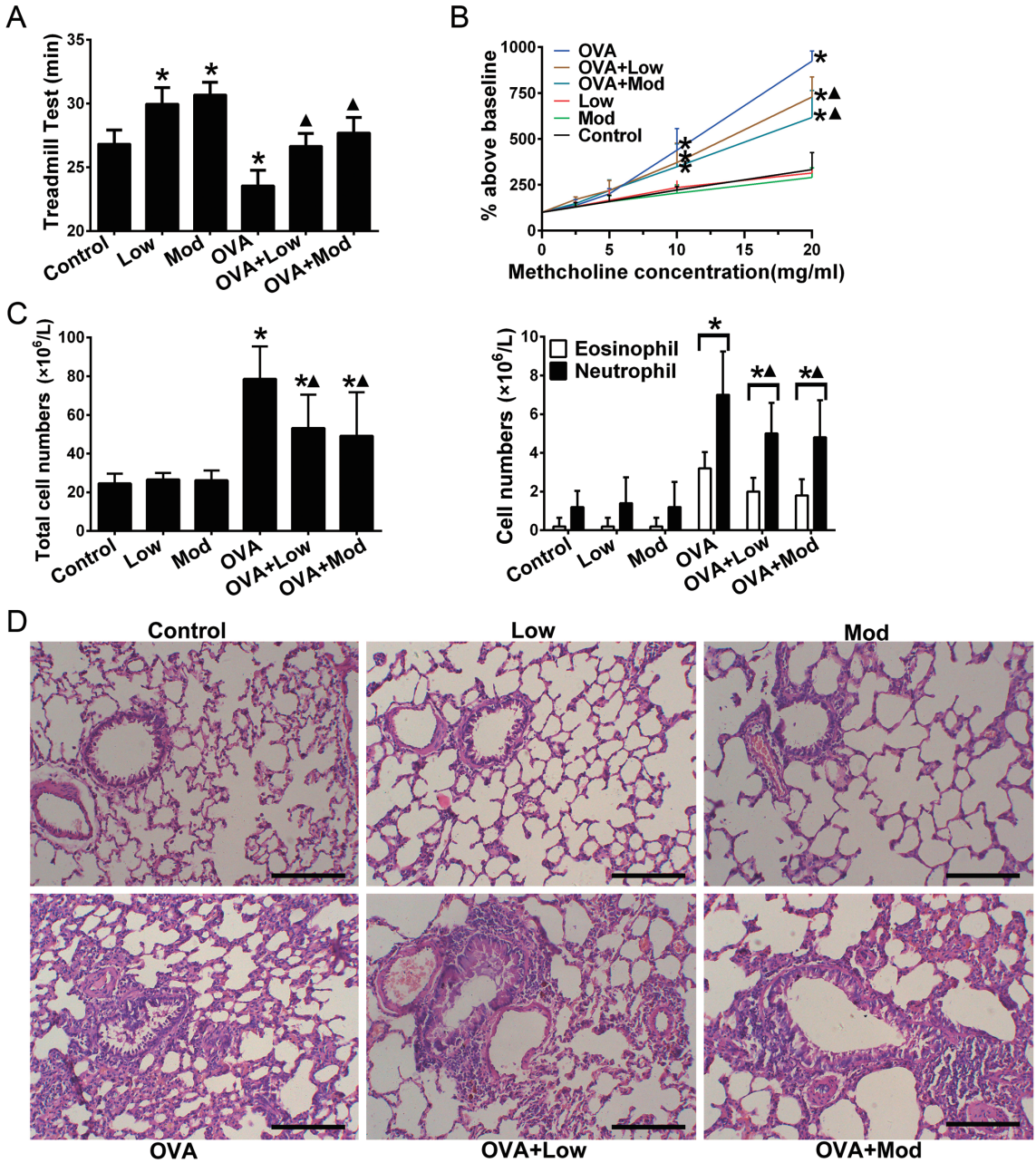

Fig. 2. Low- or moderate-intensity aerobic exercise mitigates EIB in a rat model of asthma. On day 54 after the first sensitization, individual rats were subjected to a maximal running test and the lengths of the treadmill testing period of individual rats were recorded. Two days later, the rats were challenged with a single bout of 8-min progressive exercise and then subjected to pulmonary auscultation. After that, airway responsiveness was tested by whole-body plethysmography. The airway resistance of individual rats was calculated by dividing the driving pressure by the rate of air flow (P/V). Subsequently, the BALF samples were recovered for cell counts and lung tissue sections were examined by H & E staining. Data are representative images or expressed as the mean ± S.D. of individual groups (n = 5-8 per group). (A) The lengths of final maximal running periods. (B) Changes in airway resistance in response to increasing concentration of methacholine in rats. (C) Cell counts in BALF. (D) Histological analysis of the lung (magnification × 200, scale bars = 200 μm). Control: Control rats; Low: The rats with low-intensity aerobic exercise; Mod: The rats with moderate-intensity aerobic exercise; OVA: The rats were sensitized and challenged with OVA; OVA + Low: The rats were sensitized and challenged with OVA and subjected to low-intensity aerobic exercise; OVA + Mod: The rats were sensitized and challenged with OVA and subjected to moderate-intensity aerobic exercise. *P < 0.05 vs. the control group; ▲P < 0.05 vs. the OVA group.

obvious inflammatory infiltrate in the lungs of healthy rats, while there were remarkable numbers of inflammatory infiltrates surrounding the airway in the OVA group of asthmatic rats (Fig. 2D). Notably, the numbers of inflammatory infiltrates in the OVA + Low and OVA + Mod groups of rats were reduced, as compared with that in the OVA group.

Table 2. The distribution of breathing sounds.

	Before exercise challenge				After exercise challenge			
	0	1	2	Total score	0	1	2	Total score
Control	8	0	0	0	8	0	0	0
Low	8	0	0	0	8	0	0	0
Mod	8	0	0	0	8	0	0	0
OVA	7	1	0	1	0	1	7	$15^{\#}$*
OVA + Low	8	0	0	0	4	3	1	$5^{\#}$*▲
OVA + Mod	7	1	0	1	5	2	1	4*▲

Breathing sounds were semi-quantitatively classified as follows: 0, clear breath sounds with no abnormality; 1, increased breath sounds without elements of tone or pitch; 2, increased breathe sounds with elements of tone or pitch.

Control, control rats; Low, The rats with low-intensity of aerobic exercise; Mod, The rats with moderate-intensity of aerobic exercise; OVA, The rats were sensitized and challenged with OVA; OVA + Low, The rats were sensitized and challenged with OVA and subjected to low-intensity of aerobic exercise; OVA + Mod, The rats were sensitized and challenged with OVA and subjected to moderate-intensity of aerobic exercise.

$^{\#}P < 0.05$ vs. before exercise challenge; *$P < 0.05$ vs. the control group; ▲$P < 0.05$ vs. the OVA group.

Therefore, low- or moderate-intensity aerobic exercise mitigated abnormal pulmonary auscultation, airway resistance, and inflammation in asthmatic rats, and increased the exercise capacity in both healthy and asthmatic rats.

Low- or moderate-intensity aerobic exercise improves pathological changes and endocrine function of AMCCs in asthmatic rats

Our previous studies have shown that high-intensity aerobic exercise enhances pathological changes and endocrine dysfunction of AMCCs and reduces the levels of circulating epinephrine in asthmatic rats (He et al. 2013). To understand the protective effect of low- and moderate-intensity aerobic exercise, the structure of AMCCs, and the relative levels of peripherin, neurofilament, and chromogranin A in the adrenal medulla in the different groups of rats were characterized by light microscopy, transmission electron microscopy and Western blot, respectively. Histological examination of AMCCs revealed vacuolar degeneration, increased lipid contents and blood sinus expansion under a light microscope in asthmatic rats (Fig. 3A). Electronic microscopy indicated that the density of chromaffin granules was reduced in AMCCs of the OVA group of rats, related to that in the controls ($P < 0.05$, Fig. 3B). In contrast, the morphological changes in the adrenal medullary tissues were obviously reduced in both the OVA + Low and OVA + Mod groups of rats. Furthermore, there were 6/32 (18.75%) of cells displaying neurites, a feature of neuronal transdifferentiation of AMCCs, in the OVA group, but not detectable in other groups of rats.

Peripherin and neurofilament are markers of neurons and rarely expressed in healthy AMCCs. Chromogranin A, the neuroendocrine marker protein, is the major soluble protein in the core of catecholamine storage vesicles of AMCCs. Western blot analysis showed that there was no significant difference in the relative levels of peripherin and neurofilament expression among the different groups of healthy rats and the relative levels of chromogranin A in the Low or Mod group were significantly higher than that in the control group of rats ($P < 0.05$, Fig. 3C). In comparison with that in the control group, significantly higher levels of peripherin and neurofilament and lower levels of chromogranin A expression were detected in the adrenal medulla of all the OVA group of rats. The relative levels of peripherin and neurofilament were significantly reduced, while the relative levels of chromogranin A were elevated in in the adrenal medullary tissues of the OVA + Low and OVA + Mod groups of rats, relative to that in the OVA group ($P < 0.05$).

To study the effects of aerobic exercise on the endocrine function of the adrenal medullary cells, the levels of serum epinephrine and the relative levels of PNMT expression in the adrenal medulla of individual rats were measured by ELISA and Western blot, respectively. The concentrations of circulating epinephrine in the Low and Mod groups of healthy rats were significantly higher than that in the control groups ($P < 0.05$, Fig. 4A). The concentrations of serum epinephrine in the OVA + Low and OVA + Mod groups of rats were significantly higher than that in the OVA group of asthmatic rats ($P < 0.05$). Similarly, the relative levels of PNMT expression in the adrenal medullary tissues of the Low and Mod groups of rats were significantly higher than that in the control group ($P < 0.05$, Fig. 4B). The relative levels of PNMT in the OVA + Low and OVA + Mod groups of rats were significantly higher than that in the OVA group of rats ($P < 0.05$ for all).

Collectively, these data indicated that low- or moderate-intensity aerobic exercise mitigated asthma-related pathological changes and dysfunction of AMCCs in the adrenal medullary tissues and increased the levels of serum epinephrine in both healthy and asthmatic rats, which may contribute to the improvement of lung function and exercise capacity in asthmatic rats.

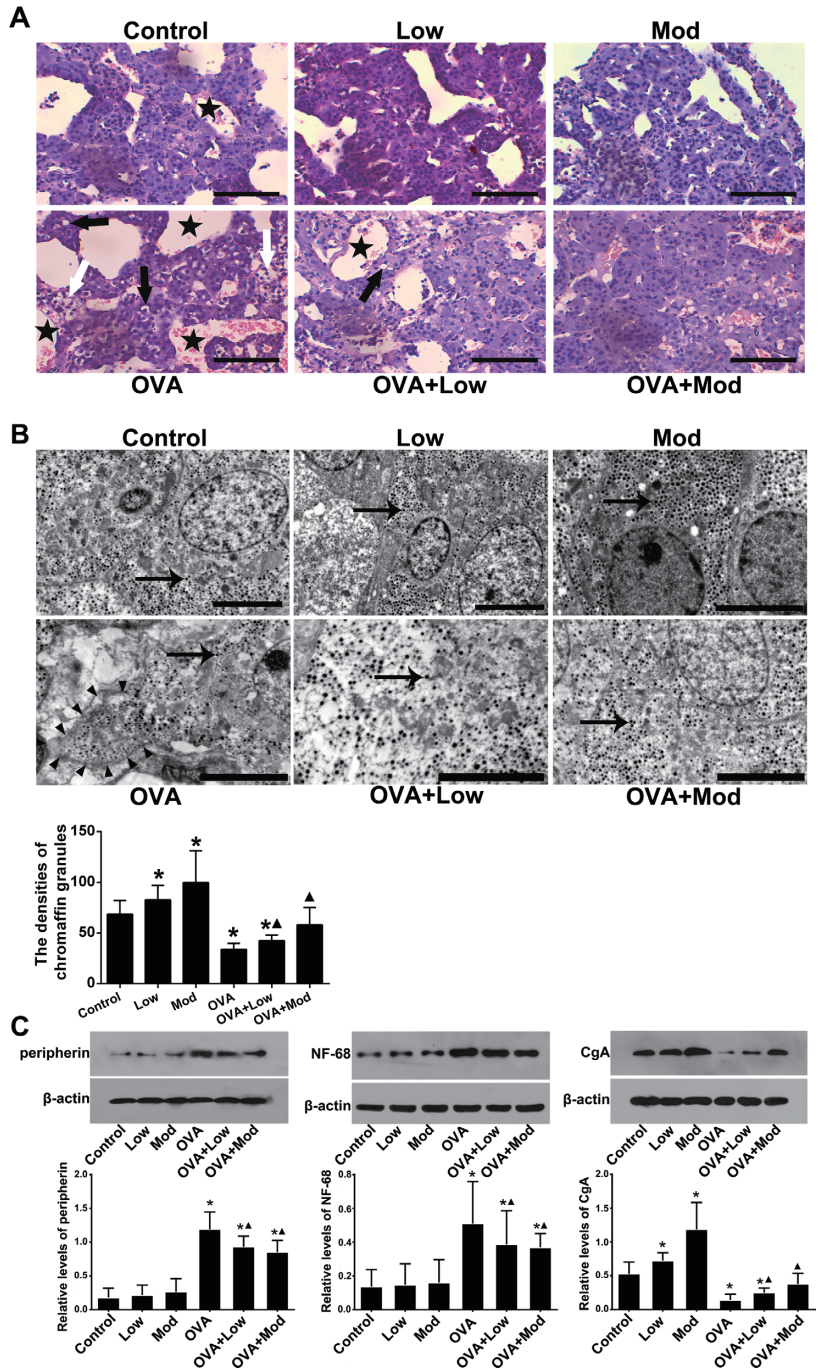

Fig. 3. Low- or moderate-intensity aerobic exercise improves pathologically morphological alterations of AMCCs in asthmatic rats.

The adrenal medullary sections of individual rats were analyzed by light microscopy and transmission electron microscopy and the relative levels of peripherin, neurofilament-68, and chromogranin A in the adrenal medulla were characterized by Western blot. Data are representative images or expressed as the mean ± S.D. of individual groups (n = 4-7 per group) from four separate experiments. (A) Morphological images (magnification × 400, scale bars = 250 μm). The arrows show vacuolar degeneration (white) and lipid (black), respectively. Star shows blood sinus (★). (B) Transmission electron microscopy analysis of AMCCs (scale bars = 5 μm). The bar graphs demonstrate quantitative analysis of the densities of chromaffin granules (number/μm^3). The black arrows indicate chromaffin granule in chromaffin cells. Neurite formation (bracketed by arrowheads) was observed in some chromaffin cells in rats of OVA group. (C) Western blot analysis of the levels of peripherin, neurofilament-68 (NF-68), and chromogranin A (CgA) expression in the adrenal medullary tissues of rats. Control: Control rats; Low: The rats with low-intensity aerobic exercise; Mod: The rats with moderate-intensity aerobic exercise; OVA: The rats were sensitized and challenged with OVA; OVA + Low: The rats were sensitized and challenged with OVA and subjected to low-intensity aerobic exercise; OVA + Mod: The rats were sensitized and challenged with OVA and subjected to moderate-intensity aerobic exercise. *P < 0.05 vs. the control group; ▲P < 0.05 vs. the OVA group.

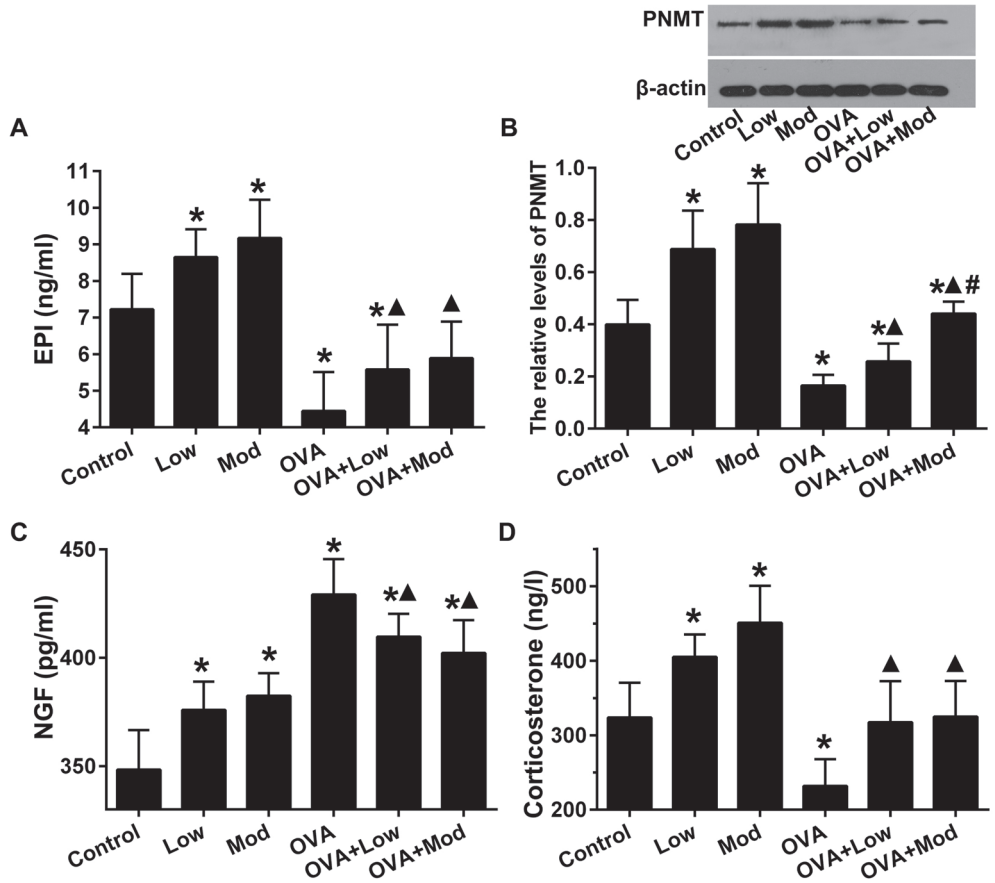

Fig. 4. Low- or moderate-intensity aerobic exercise enhances the adrenal medulla endocrine function in asthmatic rats. The levels of serum epinephrine, NGF and corticosterone in individual rats were measured by EIA, and the relative levels of PNMT in the adrenal medullary tissues were characterized by Western blot. Data are expressed as the mean ± S.D. of individual groups of rats (n = 4-7 per group) from three separate experiments. (A) The levels of serum epinephrine (EPI). (B) The relative level of PNMT expression in the adrenal medullary tissues. (C) The levels of serum NGF. (D) The levels of serum corticosterone. Control: Control rats; Low: The rats with low-intensity aerobic exercise; Mod: The rats with moderate-intensity aerobic exercise; OVA: The rats were sensitized and challenged with OVA; OVA + Low: The rats were sensitized and challenged with OVA and subjected to low-intensity aerobic exercise; OVA + Mod: The rats were sensitized and challenged with OVA and subjected to moderate-intensity aerobic exercise. *P < 0.05 vs. the control group; ▲P < 0.05 vs. the OVA group; #P < 0.05 vs. the OVA + Low group.

Low- or moderate-intensity aerobic exercise decreases the levels of serum NGF, but increases corticosterone in asthmatic rats

We next measured the levels of serum NGF and corticosterone in individual rats. Interestingly, low- or moderate-intensity aerobic exercise elevated the levels of serum NGF in healthy rats (P < 0.05) but reduced the levels of NGF in asthmatic rats (P < 0.05, Fig. 4C). In contrast, low- or moderate-intensity aerobic exercise increased the levels of serum corticosterone in healthy rats, related to that in the controls (P < 0.05, Fig. 4D) and in asthmatic rats, as compared with that in the OVA group (P < 0.05). Thus, low- or moderate-intensity aerobic exercise decreased the concentrations of serum NGF but increased corticosterone in asthmatic rats, which may improve the function of adrenal medulla in asthmatic rats.

Low- or moderate-intensity aerobic exercise mitigates asthma-related ERK/CREB activation in the adrenal medullary tissues of asthmatic rats

To understand the molecular mechanisms underlying the action of low- or moderate-intensity aerobic exercise, we characterized the relative levels of ERK and CREB phosphorylation and the relative levels of c-FOS, EGR1, c-JUN, JUNB, and FOSB mRNAs in the adrenal medullary tissues of the different groups of rats. There was no significant difference in the relative levels of ERK and CREB phosphorylation and c-FOS, EGR1, c-JUN, JUNB, and FOSB mRNAs among the healthy groups of rats, regardless of whether they underwent low- or moderate-intensity aerobic exercise (Fig. 5A, B). The relative levels of phosphorylated ERK and CREB proteins and c-FOS, EGR1, c-JUN, JUNB, and FOSB mRNAs in the adrenal medullary tissues of all the OVA group of rats were significantly higher than that in the control group (P < 0.05 for all, Fig. 5A, B).

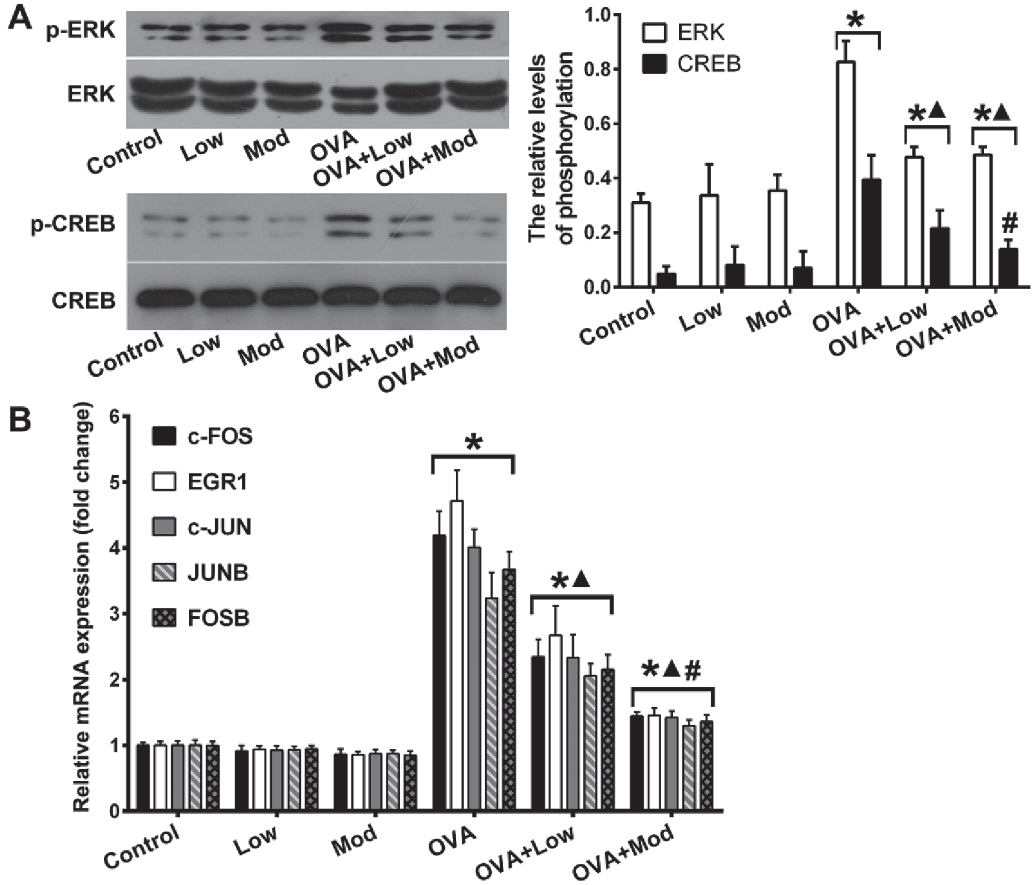

Fig. 5. Low- or moderate-intensity aerobic exercise inhibits the ERK/CREB signaling and downstream gene expression in rat adrenal medulla.

The relative levels of phosphorylated ERK and CREB were characterized by Western blot, and the relative levels of c-FOS, EGFR1, c-JUN, JUNB, and FOSB mRNAs to the control β-actin in the adrenal medullary tissues of individual rats were characterized by qRT-PCR. Data are representative images or expressed as the mean ± S.D. of individual groups of rats (n = 4 per group) from three separate experiments. (A) The relative levels of phosphorylated ERK and CREB. (B) The relative levels of c-FOS, EGFR1, c-JUN, JUNB, and FOSB mRNAs. Control: Control rats; Low: The rats with low-intensity aerobic exercise; Mod: The rats with moderate-intensity aerobic exercise; OVA: The rats were sensitized and challenged with OVA; OVA + Low: The rats were sensitized and challenged with OVA and subjected to low-intensity aerobic exercise; OVA + Mod: The rats were sensitized and challenged with OVA and subjected to moderate-intensity aerobic exercise. $^{*}P < 0.05$ vs. the control group; $^{\blacktriangle}P < 0.05$ vs. the OVA group; $^{\#}P < 0.05$ vs. the OVA + Low group.

Furthermore, the relative levels of phosphorylated ERK and CREB proteins and c-FOS, EGR1, c-JUN, JUNB, and FOSB mRNAs in the adrenal medullary tissues of the OVA + Low and OVA + Mod groups of rats were significantly reduced, as compared with that in the OVA group ($P < 0.05$ for all); and the effect of low- and moderate-intensity aerobic exercises tended to be dose-dependent. Therefore, low- or moderate-intensity aerobic exercise mitigated the ERK and CREB activation and their downstream gene expression, which may modulate epinephrine production in the adrenal medullary tissues of asthmatic rats.

Discussion

Exercise is considered to be a double-edged sword for asthmatic patients. While over-exercise can provoke an increase in airway resistance, leading to EIB, regular exercise training has been shown to improve asthmatic symptoms in both asthmatic young kids and adult patients (Farid et al. 2005; Mendes et al. 2010; Boyd et al. 2012; Avallone and McLeish 2013). The exact mechanisms underlying the action of regular exercise training are not fully understood. Our findings from the present study provided new evidence that low- and moderate-intensity aerobic exercise mitigated asthma-related morphological changes and endocrine dysfunction of AMCCs, which at least partially contributed to an increase in the levels of serum epinephrine and alleviation of EIB in asthmatic rats.

During the process of chronic asthma, inflammatory cells infiltrate into the peribronchial compartment and promote airway remodeling and EIB development. We found that airway responsiveness to exercise challenge significantly increased in the OVA group of rats, as assessed by pulmonary auscultation, consistent with the notion that exercise is one of the most common risk factors for an asth-

matic attack. In contrast, we found that regular low- or moderate-intensity aerobic exercise training reduced the numbers of inflammatory cells in BALF, and airway responsiveness to exercise challenge and to methacholine in asthmatic rats. Our data were consistent with previous observations (Pastva et al. 2004; Vieira et al. 2007; Halwani et al. 2010; Olivo et al. 2012) and our findings suggest that low- or moderate-intensity aerobic exercise may mitigate EIB in asthmatic rats.

Our previous studies have shown ultra-structural changes in AMCCs of asthmatic rats, including vacuolar degeneration, blood sinus expansion and decreased chromaffin granules and increased lipid, accompanied by impaired endocrine function of decreased epinephrine and PNMT expression (Feng and Hu 2005; Feng et al. 2012; Hu et al. 2012a, b). These pathological changes and endocrine dysfunction in AMCCs suggest a tendency towards conversion from neuroendocrine phenotype to neuronal phenotype of AMCCs. Given that epinephrine produced by chromaffin cells is critical for regulating the movement and constriction of airway smooth muscles, targeting pathological structures and endocrine dysfunction of AMCCs, such as application of Kidney-Tonifying Recipe (one kind of traditional Chinese medicine) may be a new way for the treatment of asthma (Hu et al. 2012b). In this study, we found that low- or moderate-intensity aerobic exercise mitigated pathological structural changes in AMCCs of asthmatic rats. Evidentially, low- and moderate-intensity aerobic exercise reduced vacuolar degeneration, lipid contents, but increased chromaffin granule density in the chromaffin cells, accompanied by eliminating the neurite-bearing cells in the adrenal medullary tissues of asthmatic rats. Second, low- and moderate-intensity aerobic exercises significantly reduced the levels of peripherin and neurofilament-68 expression, but increased the levels of chromogranin a expression in the adrenal medullary tissues of asthmatic rats. Furthermore, low-intensity aerobic exercise also improved the endocrine function of adrenal medulla by increasing the levels of PNMT expression and circulating epinephrine in asthmatic rats. As the PNMT is predominantly expressed by the adrenergic chromaffin cells in the adrenal medullary tissues (Evinger et al. 2007), the enhanced PNMT expression in the adrenal medullary tissues was at least partially attributed to the inhibition of neuronal transdifferentiation of chromaffin cells in the exercise-trained asthmatic rats. These data were partially consistent with our previous observations that moderate-intensity of exercise decreased peripherin expression in the adrenal medullary tissues and increased circulating epinephrine levels in asthmatic rats (He et al. 2013). However, our previous study and those of others showed that aerobic exercise training (50-75% of maximum velocity) only increased slightly the PNMT expression in the adrenal medullary tissues of healthy animal, but the increased expression was not significantly different from that in the control animals, as detected by immunohistochemistry (Bartalucci et al. 2012; He et al. 2013). Moreover, our previous study did not observe that moderate-intensity aerobic exercise has any effect on PNMT expression in the adrenal medullary tissues of asthmatic rats, as detected by immunohistochemistry (He et al. 2013). In addition, high-intensity endurance exercise decreased the levels of PNMT expression in the adrenal medullary tissues of healthy animals in our previous study (He et al. 2013), but significantly enhanced in another study (Bartalucci et al. 2012). The different results may stem from varying animal species, treatments, detection time and methods between our and their studies. NGF can promote the development of allergic inflammation and asthma via the mechanism of neurogenic inflammation (Nassenstein et al. 2006). Moreover, NGF is a potent inducer of neuronal differentiation of rat chromaffin cells (Tischler et al. 1993), which is negatively regulated by corticosterone (Unsicker et al. 1978). In this study, we observed that low- or moderate-intensity aerobic exercise reduced the levels of serum NGF, but increased the levels of serum corticosterone, accompanied by inhibiting the neuronal transdifferentiation of AMCCs in asthmatic rats. The complete block of neuronal transdifferentiation of AMCCs by low-intensity exercise may stem from elevated levels of serum corticosterone because corticosterone can prevent the neurite outgrowth from AMCCs (Unsicker et al. 1978). Interestingly, we observed that low-intensity aerobic exercise increased the levels of serum NGF in healthy rats, but decreased it in asthmatic rats. The different effects may be because exercise increased the levels of serum corticosterone, which inhibited lung inflammation, leading to a reduction of NGF secretion by activated inflammatory cells. In addition, the increased levels of serum corticosterone may also up-regulate the PNMT expression in AMCCs because corticosterone is crucial for the maintenance of PNMT expression (Jiang et al. 1989; Wan and Livett 1989). Alternatively, it is possible that low- or moderate-intensity exercise may positively regulate the sympathoadrenal system and hypothalamic-pituitary-adrenal axis, which enhance corticosterone production and the PNMT expression in AMCCs because both systems are crucial for the maintenance of circulating corticosterone (Lemaire et al. 1993; Stachowiak et al. 1988). We are interested in further investigating the potential mechanisms by which aerobic exercise regulates the PNMT expression in the adrenal medullary tissues. Nevertheless, our data indicated that low- or moderate-intensity aerobic exercise mitigated asthma-related pathologically structural changes and endocrine dysfunction in the adrenal medulla and enhanced endocrine function of AMCCs by producing more epinephrine, thereby leading to bronchodilatation in asthmatic rats. Therefore, our findings may provide a new explanation for why low- or moderate-intensity aerobic exercise mitigates EIB in asthmatic subjects.

Previous studies have indicated that NGF can promote neuronal transdifferentiation of PC12 cells in vitro (Greene and Tischler 1976; Lee et al. 1977). NGF binds to its recep-

tor and activates the ERK/CREB pathway, leading to the downstream c-FOS, EGR1, c-JUN, FOSB, and JUNB expression to promote neurite outgrowth in PC12, and treatment with the ERK-specific inhibitor of U0126 inhibits NGF-induced and aripiprazole-enhanced neurite outgrowth (Harada et al. 2001; Pellegrino and Stork 2006; Eriksson et al. 2007; Ravni et al. 2008; Chung et al. 2010; Mullenbrock et al. 2011; Ishima et al. 2012; Kudo et al. 2013). Similarly, artemisinin and its derivatives induce neurite outgrowth of PC12 cells in an ERK/CREB dependent manner (Sarina et al. 2013). We found that low-intensity aerobic exercise inhibited the ERK and CREB phosphorylation and c-FOS, EGR1, c-JUN, FOSB, and JUNB expression in the adrenal medullary tissues of asthmatic rats. These findings were consistent with our previous observations following moderate-intensity exercise training in asthmatic rats although high-intensity endurance exercise enhanced the ERK activation in the adrenal medullary tissues of asthmatic rats (He et al. 2013). However, both low- and moderate-intensity exercise trainings had no significant effect on the ERK activation in the adrenal medullary tissues of healthy rats. The lack of enhanced ERK activation in healthy rats may be because low- or moderate-intensity exercise elevated serum glucocorticoid, which attenuated the effects of NGF on the ERK activation in the adrenal medullary tissues of healthy rats. Given that the ERK/CREB signaling plays an important role in the phenotypic transformation of AMCCs, our findings suggest that the inhibition of neuronal transdifferentiation of AMCCs by low- or moderate-intensity aerobic exercise may be associated with inhibiting the ERK/CREB activation and downstream gene expression in the adrenal medulla of asthmatic rats. It is possible that low- or moderate-intensity aerobic exercise promotes corticosterone production, which inhibits the ERK/CREB activation by antagonizing the effect of NGF or indirectly through the crosstalk between the corticosterone's nuclear receptor-mediated signaling and the NGF-mediated ERK signaling in AMCCs. We are interested in further examining the molecular mechanisms by which low or moderate intensity aerobic exercise down-regulates the ERK/CREB signaling in AMCCs.

In conclusion, low-intensity aerobic exercise mitigated EIB and increased the exercise capacity of asthmatic rats. Furthermore, low-intensity aerobic exercise improved morphological changes and endocrine dysfunction of AMCCs, thereby leading to an increase in the levels of serum epinephrine in asthmatic rats. In addition, low-intensity aerobic exercise reduced the levels of serum NGF, but increased the levels of serum corticosterone as well as inhibited the ERK/CREB phosphorylation and downstream gene expression in the adrenal medullary tissues of asthmatic rats. Therefore, our findings may provide new insights into the mechanisms underlying the action of low-intensity aerobic exercise in regulating not only inflammation, but also the endocrine function of AMCCs. Conceivably, our findings may explain the beneficial effect of regular exercise on asthmatic patients.

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Conflict of Interest

The authors declare no conflict of interest.

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