High-intensity training induces EIB in rats through neuron transdifferentiation of adrenal medulla chromaffin cells

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High-intensity training induces EIB in rats through neuron transdifferentiation of adrenal medulla chromaffin cells. Am J Physiol Lung Cell Mol Physiol 304: L602–L612, 2013. First published February 15, 2013; doi:10.1152/ajplung.00406.2012.—A high prevalence of exercise-induced bronchoconstriction (EIB) can be found in elite athletes, but the underlying mechanisms remain elusive. Airway responsiveness, NGF and epinephrine (EPI) levels, and chromaffin cell structure in high- (HiTr) and moderate-intensity training (MoTr) rats with or without ovalbumin (OVA) sensitization were measured in a total of 120 male Sprague-Dawley rats. The expression of NGF-associated genes in rat adrenal medulla was tested. Both HiTr and OVA intervention significantly increased airway resistance to aerosolized methacholine measured by whole body plethysmography. HiTr significantly increased inflammatory reaction in the lung with a major increase in peribronchial lymphocyte infiltration, whereas OVA significantly increased the infiltration of various inflammatory cells with an over 10-fold increase in eosinophil level in bronchoalveolar lavage. Both HiTr and OVA intervention upregulated circulating NGF level and peripherin level in adrenal medulla, but downregulated phenylethanolamine N-methyltransferase level in adrenal medulla and circulating EPI level. HiTr + OVA and HiTr + ExhEx (exhaustive exercise) interventions significantly enhanced most of the HiTr effects. The elevated NGF level was significantly associated with neuronal conversion of adrenal medulla chromatil cells (AMCC). The levels of p-Erk1/2, JMD3, and Mash1 were significantly increased, but the levels of p-p38 and p-JNK were significantly decreased in adrenal medulla in HiTr and OVA rats. Injection of NGF antiserum and moderate-intensity training reversed these changes observed in HiTr and/or OVA rats. Our study suggests that NGF may play a vital role in the pathogenesis of EIB by inducing neuron transdifferentiation of AMCC via MAPK pathways and subsequently decreasing circulating EPI.

EXERCISE-INDUCED BRONCHOCONSTRICTION (EIB) is the increase in airway obstruction with symptoms of coughing, wheezing, or dyspnea as a result of exercise (23). EIB occurs in ~11% of the general population without asthma (13). Inhalation of cold air and sudden changes in humidity or temperature have been associated with EIB (20). High prevalence of EIB is usually observed among endurance athletes (7), implicating that intensity of exercise may be associated with EIB.

Although thermal and osmotic consequences of water loss play prominent roles in the emergence of EIB (2), more studies are needed to elucidate the underlying mechanism. Circulating epinephrine (EPI), produced by adrenal medulla chromaffin cells (AMCC), is believed to bind the adrenergic receptors of airway smooth muscles to achieve adrenergic bronchodilation, and a loss of this function could directly influence the severity of the obstruction. In a previous study, circulating EPI was found to be reduced in EIB (3), and inhalation of β2-adrenoceptor agonists was able to effectively reverse EIB (7). Our previous study in rats revealed that EPI levels are decreased in asthmatic rats, and this decrease is associated with a phenotype transition from AMCC to neurons (12). However, which mechanisms drive this phenotype transition remains unclear. Sympathetic adrenal cells, derived from embryonic neural crest stem cells, are able to migrate and relocate to the adrenal gland during their development. After losing their neuronal features, these cells differentiate into AMCC with endocrine function. A previous study demonstrated that AMCC can be converted to neurons, which are associated with the activation of mitogen-activated protein kinase (MAPK) pathway (26). JMD3 is a specific histone H3 lysine 27 demethylase (1), which controls the expression of key regulators and markers of neurogenesis (6). Mash1 is a basic helix-loop-helix regulatory protein that plays a critical role in neurogenesis, which could be the target of JMD3 (10). Moreover, the expression of JMD3 is regulated by ERK/MAPK signaling (24). We hypothesize that ERK-JMD3-Mash1 signaling may be involved in the transition of AMCC to neurons. Importantly, the conversion of AMCC to neurons is accompanied by elevated nerve growth factor (NGF) level and a lack of glucocorticoids (31). Likewise, upregulation of NGF level during exercise has been demonstrated previously (8). We further hypothesize that insufficient circulating EPI levels are caused by NGF-mediated neuron transition of chromaffin cells during exercise. The lack of circulating EPI may play a key role in the pathogenesis of EIB through activation of ERK-JMD3-Mash1 signaling.

In contrast to EIB, which is associated with strenuous exercise (such as long-distance running, swimming, and weight lifting), aerobic training has been used as an important part of the rehabilitation regimen for asthmatic patients, which can decrease dyspnea, airway hyperresponsiveness, exercise-induced bronchospasm, and use of corticosteroids and can improve aerobic capacity and quality of life (11). Obviously, distinct exercise intensities play different roles in airway responsiveness. In this study, we comparatively investigated airway responsiveness, the levels of circulating NGF and EPI, structural changes of chromaffin cells in moderate- and high-intensity training rats with or without ovalbumin (OVA) sensitization, as well as the role of a bout of exhaustive exercise and NGF antiserum injection on these changes and their molecular mechanisms.

MATERIALS AND METHODS

Experimental animals. All studies were approved by the Institutional Animal Care and Use Committee of Xiangya School of Medicine, Central South University. All animal procedures and experi-
mental protocols complied with NIH guidelines. Male Sprague-Dawley rats (6 wk old, weight range 180–200 g) were housed under a quiet and antigen-free environment, with standardized temperature and humidity and free access to food and water.

Maximal running test and exercise protocol. All rats were subjected to the maximal running test to measure maximum aerobic velocity (MAV) (28), an index reflecting the maximum oxygen consumption (\(V\dot{O}_{2\text{max}}\)), and to determine a moderate-intensity or high-intensity training regimen based on their \(V\dot{O}_{2\text{max}}\) values. The highest velocity recorded before the animal was unable or unwilling to continue was considered the MAV. After MAV determination, rats were subjected to a moderate- (speed at 25 m/min, 65–70% of maximal speed) or high-intensity training regimen (speed at 35 m/min, 85–90% of maximal speed) for 60 min a day, 6 days a week for 8 wk (4 wk before OVA sensitization + 4 wk after OVA or saline exposure). The treadmill speed was gradually increased over the course of the 8-wk training period. To establish a protocol for exhaustive exercise, rats were placed on a treadmill beginning at 15 m/min, and the speed was increased gradually until it reached a final speed of 35 m/min. Exhaustion was defined as the loss of a rat’s righting reflex due to extensive exercise (the rat being unable to upright itself when placed on its back) as previously described with little modification (17). The time required for rats to reach exhaustion was used to verify the efficacy of different intensity training regimen. All sedentary rats were exposed to treadmill exercise three times before the exhaustive exercise to acclimate them to running on the treadmill.

One hundred twenty rats were randomly divided into 15 groups: 1) control group; 2) exhaustive exercise group (ExhEx group); 3) moderate-intensity training group (MoTr group); 4) MoTr + NGF.
antiserum group (MoTrN group); 5) MoTr + exhaustive exercise group (MoTr + ExhEx group); 6) MoTr + NGF antiserum + exhaustive exercise group (MoTrN + ExhEx group); 7) high-intensity training group (HiTr group); 8) HiTr + NGF antiserum group (HiTrN group); 9) HiTr + exhaustive exercise group (HiTr + ExhEx group); 10) HiTr + NGF antiserum + exhaustive exercise group (HiTrN + ExhEx group); 11) OVA-treated group (OVA group); 12) OVA-treated + MoTr group (OVA + MoTr group); 13) OVA-treated + MoTr + NGF antiserum group (OVA + MoTrN group); 14) OVA-treated + HiTr group (OVA + HiTr group); 15) OVA-treated + HiTr + NGF antiserum group (OVA + HiTrN group) (n = 8 per group). Thirty minutes before training each day, NGF antiserum (1:1,000 dilution, 4 ml·kg⁻¹·day⁻¹, R&D Systems, Minneapolis, MN) (33) or PBS (4 ml/kg) was injected intraperitoneally in rats. Electric shock was only used during acclimation training to make the rats start running.

Because of the large number of groups examined in this study, the data of several groups are presented together to reflect a comparison. For example, MoTr, MoTrN, HiTr, and HiTrN groups are presented in one figure to reflect the mechanisms of different training intensities in EIB. ExhEx, MoTr + ExhEx, MoTrN + ExhEx, HiTr + ExhEx, and HiTrN + ExhEx groups are presented in one figure to reflect the impact of exhaustive exercise. OVA, OVA + MoTr, OVA + MoTrN, OVA + HiTr, and OVA + HiTrN groups are presented in one figure to show the interaction of OVA intervention with different exercise intensities in the mechanisms of EIB.

OVA sensitization. The antigen sensitization period began on the fifth week of physical training and lasted 4 wk. The rats were sensitized by an intraperitoneal injection of 100 mg of OVA (grade V) (Sigma-Aldrich, St. Louis), 100 mg of aluminum hydroxide (Sigma), and 6 × 10⁵ heat-killed Bordetella pertussis (Wuhan Institute of Biological Products, Wuhan, China) in 1 ml of sterile saline on days 28 and 42 (9) or with saline (NaCl 0.9%) in the sedentary control rats. On day 49, animals were challenged with 1% OVA (wt/vol) aerosol via an ultrasonic nebulizer (Boehringer-Ingelheim, Ingelheim, Germany) for 30 min/day every other day for a total of five rounds. Whereas the control rats were exposed to aerosolized sterile saline only, exercised rats underwent rounds of exercise on a treadmill 1 h following OVA intervention. The design of the treadmill training protocol and OVA intervention is shown in Fig. 1.

Measurement of airway responsiveness. Rats were anesthetized by intraperitoneally injection of 10% chloral hydrate solution (3.5 ml/kg). Invasive single-chamber plethysmography was used to assess airway resistance. After a round of exhaustive exercise or the last OVA challenge, invasive measurement of pulmonary mechanics was conducted in rats in response to methacholine as previously described (21): 1) the thorax was opened; 2) rats were ventilated with a tidal volume of ~5–6 ml/kg and respiratory rate of 90 breaths/min by use of a MiniVent Ventilator for small animals (Harvard Apparatus, Holliston, MA); 3) rats received aerosolized solutions of methacholine (0.25, 5.0, 10, and 20 mg/ml in normal saline) (22); 4) a commercial plethysmography system (model PLY3211 plethysmograph, MAX II...
amplifier and pressure transducer system, and Biosystem XA software, Buxco Electronics) was used to quantify airway resistance (Rt). Rt is found by dividing the driving pressure by rate of air flow. Results for each methacholine concentration were expressed as the percentage of baseline Rt values after saline exposure.

*Bronchoalveolar lavage and cell counting.* After measurement of airway responsiveness, blood was collected by cardiac puncture and rats were euthanized. Afterward, the right main stem bronchus was occluded with a clamp and the left lobe of the lung was lavaged in situ with three successive 3-ml volumes of saline instilled by a syringe. Bronchoalveolar lavage fluid (BALF) was centrifuged at 1,000 rpm for 10 min at 4°C. The sediment was then stained with May-Grunwald-Giemsa and a total of 300 cells were counted (33).

*Hematoxylin and eosin staining and immunohistochemistry.* The right middle lung lobe and adrenal medulla of rats were fixed in 4% paraformaldehyde and then embedded in paraffin. Tissue sections (3–4 μm) were stained with HE, and the morphological changes of the lung and adrenal medulla were observed under a light microscope (Leica Microsystems, Wetzlar, Germany). For immunohistochemical analysis, sections were stained with polyclonal rabbit antibodies to phenylethanolamine N-methyl transferase (PNMT) and peripherin (anti-PNMT, 1:2,500, Millipore; anti-peripherin, 1:2,000, Abcam), by use of an ABC kit and DAB substrate kit (Zhongshang Biologic, Beijing, China). The results were observed under a light microscope (×400), and the average optical density was calculated with use of the Image Pro Plus 6.0 software (IPP6.0) for subsequent statistical analysis.

*Transmission electron microscopy.* Adrenal medullas were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After 3 h, specimens were fixed in buffered 1% OsO4 for 1 h, dehydrated in ethanol, and embedded in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate and finally examined under the H-7500 transmission electron microscope (Hitachi, Japan). Ten random fields were analyzed.

*Enzyme-linked immunosorbent assay.* Serum levels of EPI, NGF, and corticosterone (CORT) were measured by using ELISA kits according to the user manual (EPI: R&D Systems; NGF: Abcam, Cambridge, UK; CORT, Abnova, Walnut, CA). The reactions were read at 450 nm in a plate reader.

*Western blot analysis.* Total proteins were extracted, 30 μg of protein were separated on 8% sodium dodecyl sulfate-polyacrylamide gel, and the proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Mem-

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**Fig. 3.** Morphological changes in rat adrenal medulla chromaffin cells. A: morphological changes under light microscope. Magnification ×200. Scale bars = 100 μm. B: ultrastructure under transmission electron microscope. Black arrow points to a cell process and junction. *Inset* shows rough endoplasmic reticulum (†), which morphologically likes Nissl body in neuron cells. White arrow illustrates chromaffin granules aggregate peripherally, indicating the route of chromaffin granule discharge. Magnification ×10,000. Scale bars = 20 μm.
branes were incubated at 4°C overnight with primary antibodies against peripherin (1:20,000; Abcam), JMJD3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Mash1 (1:250; BD Biosciences, San Jose, CA), phosphorylated extracellular signal-regulated kinases (p-ERK), extracellular signal-regulated kinases (ERK), phosphorylated p38 (p-p38), p38, phosphorylated c-Jun amino-terminal kinases (p-JNK), and c-Jun amino-terminal kinases (JNK) (1: 1,000; Cell Signaling Technology, Boston, MA) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Reactions were visualized by enhanced chemiluminescence reagents (Pierce, Rockford, IL) and quantified using Glyko Bandscan 5.0 (Glyko, Novato, CA). Results were expressed as the ratio of the mean band density of experimental groups to that of the control group after normalization to β-actin as an internal control.

Real-time PCR measurement. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg of total RNA using a cDNA first-strand synthesis kit (Ferments, Vilnius, Lithuania). JMJD3 gene was amplified by the forward primer 5'-GGATGACCTCTATGCAGTCAAT-3' and reverse primer 5'-CGTTCTCAGCTGTTCCACTC-3'. Mash1 gene was amplified by the forward primer 5'-CAACAAGAGATGACGCAAGGTG-3' and reverse primer 5'-AACCAGC-CATAGAGTCAAGT-3'. β-Actin was amplified by using the forward primer 5'-TGTTACCAACTGGGAGAAC-3' and reverse primer 5'-GGGTTGTTAGGTTCCTCAGCA-3' as internal control. Amplification was performed in duplicate by using a SYBR Green PCR kit (SYBR Premix Ex Taq, Takara, Japan) in a 7900HR Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The real-time PCR programs for JMJD3, Mash1, and β-actin were initiated by a 10-s denaturation step at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and a final extension at 60°C for 15 s. Results were expressed as the ratio of the mean threshold cycle (Ct) values of experimental groups to that of the control group after normalization to β-actin.

Statistical analyses. Data were expressed as means ± SD. Statistical analysis of data was performed with SPSS 16.0 software. One-
way ANOVA followed by Student-Newman-Keuls test was used to compare the differences among multiple groups. A $P < 0.05$ was considered statistically significant.

**RESULTS**

High-intensity training and OVA sensitization provoked inflammatory reaction in lung tissues and increased airway resistance to methacholine, but moderate-intensity training and injection of NGF antiserum reversed the changes induced by high-intensity training and OVA sensitization. The $R_t$ value in the HiTr group was significantly elevated compared with the control group. $R_t$ value in the OVA group was significantly higher than in other groups. The $R_t$ values were further elevated in the HiTr + OVA and HiTr + ExhEx groups. MoTr intervention decreased $R_t$ values in the OVA group. ExhEx also significantly elevated the $R_t$ in the MoTr group. Addition of NGF antiserum was able to markedly suppress the augmentation of $R_t$ in HiTr and HiTr + OVA rats (Fig. 1B).

Under the microscope, infiltration of inflammatory cells can be seen in the lung tissue of HiTr rats. Thickening of the airway epithelium, peribronchial inflammatory cell infiltration, and smooth muscle hypertrophy were observed in the lung tissues of OVA-treated rats. These lesions were aggravated in the HiTr + OVA group and alleviated by MoTr and administration of NGF antiserum (Fig. 2A).

The infiltrated lymphocyte count in the peribronchial tissue was significantly increased in the HiTr group. A significantly higher number of various inflammatory cells were found in OVA-treated rats, whereas MoTr + OVA intervention alleviated these phenomena. On the other hand, HiTr + OVA intervention tended to attenuate them. The influx of inflammatory cells was much lower with injection of NGF antiserum in both the HiTr + ExhEx group and HiTr + OVA group (Fig. 2B).

High-intensity training and OVA sensitization stimulate conversion of AMCC to neurons in rat adrenal medulla and are associated with elevated circulating NGF and decreased EPI levels. After HiTr, examination of AMCC revealed a disordered structure, vacuolar degeneration, and increased lipid content under light microscope (Fig. 3A). Under electron microscopy, AMCC in HiTr group exhibited edematous cytoplasm, decreased chromaffin granules, and fiber outgrowth. Also, AMCC exhibited some features of neuron cells, including cell processes and junctions and increased rough endoplasmic reticulum and mitochondrion density (Fig. 3B). The morphological changes were aggravated in OVA-treated rats. Exposure of OVA-treated rats to MoTr was able to alleviate these lesions, whereas exposure to HiTr tended to exacerbate them. Addition of NGF antiserum alleviated these changes to some extent (Fig. 3). The adrenal volume increased after a period of...
strenuous training, which is consistent with previous studies (4), and was accompanied by increases in cell size, swollen cytoplasm, and/or extracellular space as well as interstitial fiber.

Peripherin is a marker of peripheral neurons and mainly expressed in the cytoplasm of neurons (14). Peripherin is rarely expressed in normal AMCC. Peripherin expression, detected by immunostaining (Fig. 4A) and Western blot (Fig. 4, B and C), was significantly elevated in HiTr and OVA groups. The augmentation effect of OVA intervention on peripherin expression was inhibited by MoTr but enhanced by HiTr. However, the elevated peripherin level in rats decreased when injected with NGF antiserum (Fig. 4).

PNMT is primarily expressed in AMCC but absent in neurons. Positive staining of PNMT, regarded as a feature of epinephrine cells (26), was distributed throughout the cytoplasm of rat adrenal medulla cells. The intensity of PNMT immunostaining was markedly attenuated in HiTr and HiTr + ExhEx groups, which diminished greatly after OVA intervention. Enhanced expression of PNMT was observed in AMCC from HiTrN and HiTrN + ExhEx groups treated with NGF antiserum (Fig. 5).

We further measured serum EPI, NGF, and CORT levels. Both HiTr and OVA intervention downregulated EPI but upregulated NGF level in rats. Injection of NGF antiserum tended to reduce EPI and NGF levels in HiTr and OVA-treated rats. Exhaustive exercise significantly elevated the levels of EPI and CORT, but after high-intensity training, exhaustive exercise was not able to increase their levels. CORT level was not significantly elevated after HiTr and decreased with OVA intervention (Table 1).

<table>
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<tr>
<th>Group</th>
<th>EPI, ng/ml</th>
<th>NGF, ng/l</th>
<th>CORT, ng/l</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.61 ± 0.61</td>
<td>342.37 ± 30.66</td>
<td>322.07 ± 35.27</td>
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<tr>
<td>ExhEx</td>
<td>10.14 ± 0.77</td>
<td>370.63 ± 21.28</td>
<td>458.27 ± 53.94</td>
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<td>MoTr</td>
<td>6.21 ± 0.97</td>
<td>317.40 ± 31.26</td>
<td>509.86 ± 58.04</td>
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<tr>
<td>MoTrN</td>
<td>6.44 ± 0.63</td>
<td>283.43 ± 19.45</td>
<td>498.95 ± 62.72</td>
</tr>
<tr>
<td>HiTr</td>
<td>9.55 ± 0.69</td>
<td>320.41 ± 23.73</td>
<td>583.47 ± 52.10</td>
</tr>
<tr>
<td>HiTrN</td>
<td>9.61 ± 1.26</td>
<td>298.84 ± 26.68</td>
<td>521.01 ± 43.78</td>
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<tr>
<td>HiTrN + ExhEx</td>
<td>2.42 ± 0.59</td>
<td>410.35 ± 37.17</td>
<td>432.43 ± 74.16</td>
</tr>
<tr>
<td>HiTrN + ExhEx</td>
<td>4.97 ± 0.92</td>
<td>309.90 ± 32.84</td>
<td>485.05 ± 37.00</td>
</tr>
<tr>
<td>HiTrN + ExhEx</td>
<td>6.08 ± 0.97</td>
<td>305.61 ± 23.17</td>
<td>435.87 ± 86.43</td>
</tr>
<tr>
<td>OVA</td>
<td>2.14 ± 0.58</td>
<td>499.07 ± 32.79</td>
<td>256.33 ± 13.45</td>
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<tr>
<td>OVA + MoTr</td>
<td>4.08 ± 0.50</td>
<td>358.38 ± 39.25</td>
<td>320.71 ± 51.47</td>
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<tr>
<td>OVA + MoTrN</td>
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<td>283.56 ± 22.37</td>
<td>305.24 ± 24.26</td>
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<tr>
<td>OVA + HiTr</td>
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<td>486.56 ± 44.69</td>
<td>314.58 ± 18.92</td>
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<tr>
<td>OVA + HiTrN</td>
<td>3.30 ± 0.85</td>
<td>314.86 ± 27.18</td>
<td>369.79 ± 24.27</td>
</tr>
</tbody>
</table>

Values are means ± SD (*n* = 8 rats/group). EPI, epinephrine; NGF, nerve growth factor; CORT, corticosterone. Groups were control group; exhaustive exercise group (ExhEx); moderate-intensity training group (MoTr); MoTr + NGF antiserum group (MoTrN); MoTr + NGF antiserum + exhaustive exercise group (MoTr + ExhEx); high-intensity training group (HiTr); HiTr + NGF antiserum group (HiTrN); HiTr + exhaustive exercise group (HiTr + ExhEx); HiTr + NGF antiserum + exhaustive exercise group (HiTrN + ExhEx); ovaalbumin-treated group (OVA); OVA-treated + MoTr group (OVA + MoTr); OVA-treated + MoTr + NGF antiserum group (OVA + MoTrN); OVA-treated + HiTr group (OVA + HiTr); OVA-treated + HiTr + NGF antiserum (OVA + HiTrN). *p* < 0.05 vs. HiTr; **p** < 0.05 vs. Control; ***p** < 0.05 vs. MoTr + ExhEx; *p* < 0.05 vs. HiTr + ExhEx; *p* < 0.05 vs. OVA; *p* < 0.05 vs. OVA + MoTr; *p* < 0.05 vs. OVA + HiTr.

**High-intensity training and OVA sensitization regulated NGF-associated gene expression in rat adrenal medulla.** As shown in Fig. 6, exposure of rats to high-intensity exercise or OVA significantly decreased the levels of p-p38 and p-JNK. On the other hand, a significant increase in p-ERK level was observed in HiTr and OVA-treated rats. MoTr alleviated these changes in OVA-treated rats, but HiTr attenuated it. ExhEx tended to elevate the expression of p-ERK but did not have the same effect on p-p38 and p-JNK. Injections of NGF antiserum exhibited a significant ameliorating effect on the expression of p-ERK, but not p-JNK. Significant increase in the mRNA and protein expressions of JMJD3 and Mash1 genes were observed in HiTr- and OVA-treated rats. Rats treated with OVA + HiTr exhibited a significant upregulation of JMJD3 and Mash1 expressions. Conversely, rats in the OVA + MoTr group exhibited a significant downregulation in Mash1 level compared with the OVA group (Fig. 7). Exhaustive exercise induced a significant increase in the levels of JMJD3 mRNA and protein expression in rats in MoTr + ExhEx and HiTr + ExhEx groups but had no effect on Mash1 expression. Clearly, NGF antiserum inhibited the increase in the protein expression and mRNA levels of JMJD3 and Mash1 (Fig. 7).

**DISCUSSION**

Olympic-level athletes face an increased risk of EIB, especially those who take part in endurance sports, such as swimming, running, or winter sports (7). However, numerous questions regarding EIB have not been addressed, such as why are asthmatic patients sensitive to glucocorticoid therapy but not EIB patients, what are the different mechanisms involved in EIB and aerobic training, and how are neuroendocrine mechanisms involved in EIB, etc. In this study, we investigated airway responsiveness, circulating NGF and EPI levels, the structure of chromaffin cells, and NGF-associated signaling changes in HiTr and MoTr groups with or without OVA sensitization. We highlighted the role of inflammation, NGF, and conversion of chromaffin cells to neurons in EIB.

Previous studies demonstrated that lymphocytes were increased in the BALF of cross-country skiers (27), and inflammation and airway remodeling were observed in their bronchial biopsies (19). In addition, OVA exposure dramatically elevated airway responsiveness even at low antigen concentration (16). In this study, we observed a significant increase in the infiltration of peribronchial inflammatory cells, particularly the lymphocytes in HiTr-treated rats. On the other hand, various inflammatory cells were observed in OVA-treated lung as well, especially the number of eosinophils, which was found to be elevated to 10 times the normal value. The difference in the type of infiltrated cells (lymphocytes vs. eosinophils) may explain why EIB patients are not sensitive but asthmatic patients are sensitive to glucocorticoid therapy. Importantly, we found that moderate-intensity training significantly antagonized the induction effect of OVA in inflammation. Aerobic training has been recommended as an effective adjutant intervention in the management of asthmatic patients (11). In this study, the decrease in airway responsiveness and inflammation was confirmed after MoTr intervention in OVA-sensitized rats. In contrast, HiTr exposure exerted an opposite effect and tends to increase the airway response to OVA, which may explain the high incidence of EIB among top athletes with asthma.
Intense physical exercise can be considered a stressor that should be able to stimulate the sympathoadrenal system and hypothalamic-pituitary-adrenal axis, which is reflected by the levels of EPI and CORT, respectively. Our study showed that high-intensity training reduced plasma EPI, but after a bout of submaximal exercise the level of EPI could not be increased accordingly. In contrast, MoTr could upregulate EPI and CORT levels. This could explain the occurrence of airway hyperresponsiveness among endurance athletes after competitions because the blunted sympathoadrenal system is unable to release sufficient EPI to dilate the bronchus (12, 16, 33) after prolonged high-intensity training. The time required for rats to reach exhaustion was used to evaluate the efficacy of different training regimens in inducing exercise tolerance. We found that exercise at any intensity can induce a significant increase in the latency to reach exhaustion, which suggested that training could increase exercise tolerance (5). We also found that rats in the HiTr group took longer to reach exhaustion than rats in the MoTr group (data not shown).

A novel finding in this study is the conversion of chromaffin cells to neurons in high-intensity training rats. Chromaffin cells share many molecular and structural features with sympathetic neurons, including a common origin from the neural crest (30). The developmental relationship between AMCC and sympathetic neurons implicates that early postnatal AMCC can be transdifferentiated into neurons when exposed to NGF (31). The evidence for neuron conversion in this study include 1) increased cell size and lipid content in AMCC cells as well as disordered structure, vacuolar degeneration, and loss of chromaffin granules in their cell bodies; 2) decreased PNMT level in rat adrenal medulla, which is a marker of the adrenergic phenotype (26); 3) acquisition of characteristic neuronal ultrastructure, including morphological processes and junctions, augmented rough mitochondrion, and endoplasmic reticulum (like neurons specialized Nissl body), increased euchromatin, and myelinated nerves; 4) response to a neuronal marker: elevated neurofilament protein-peripherin expression. PNMT is the final enzyme in catecholamine biosynthesis, converting norepinephrine to EPI (34). Therefore, decreased PNMT level may be responsible for the low circulating EPI level observed in HiTr and OVA-treated rats. Peripherin also plays a vital role in initiation, extension, and maintenance of neuritis (14). However, how chromaffin cells are converted to neurons underlying EIB remains unknown.

Another important finding in the present study is that the NGF level was elevated in HiTr and OVA-treated rats, but not in MoTr rats. Injection of NGF antiserum could reverse the changes of airway inflammation, and remodeling as well as
decrease airway responsiveness in HiTr and OVA-treated rats, which might implicate the importance of NGF in EIB. Previous studies demonstrated that an increase in inflammatory cells, including lymphocytes, eosinophils, and macrophages, was associated with an increase in the production of NGF (25). However, how NGF regulates the conversion of chromaffin cells to neurons remains unknown. Various in vitro studies have demonstrated that various signal molecules in MAPK pathways, including ERK, JNK, and p38 MAPKs, play a crucial role in NGF-induced neurite outgrowth from adrenal chromaffin cells (24). NGF is able to produce sustained activation of ERK in normal chromaffin cells through Trk-controlled activation of Ras-mediated MAPK cascade (26). The expression of JMJD3 is regulated by ERK signaling (24). However, the role of JMJD3 in AMCC neuronal transition has not been verified. Mash1 plays a crucial role in neurogenesis mainly in the early development of neural and neuroendocrine progenitor cells in a number of tissues including the adrenal medulla, central nervous system, and others (18). A study in undifferentiated mice embryonic stem cells demonstrated that JMJD3 could demethylate Mash1 promoter and play important roles in neuronal differentiation (32). In this study, the levels of NGF, p-ERK, JMJD3, and Mash1 were significantly increased,
but the levels of p-p38 and p-JNK were significantly decreased in HiTr and OVA-treated rats. Injection of NGF antiserum had a tendency to reverse HiTr and OVA-induced p-ERK expression but exhibited a limited role on the expression of other proteins. Therefore, elevated NGF level may regulate the conversion of chromaffin cells to neurons underlying EIB through ERK, but not p38 or JNK.

In conclusion, a mechanism involved in EIB attacks was proposed in this study: the thermal and osmotic consequences of water loss lead to inflammation and the release of mediators in the lung. The inflammatory cells or exposed nerves produce and release NGF, and the elevated circulating NGF regulates the conversion of chromaffin cells to neurons through upregulating p-ERK-JMJD3-Mash1 signaling and regulating peripheral and adrenergic expression via posttranscriptional control of the PNMT gene (29). The neuronal transition may provoke alternations in the morphology and function of AMCC, which reduce the baseline and stress-induced EPI levels and subsequently cause the ineffective dilatation of the bronchi. The enhancement of NGF and reduction of EPI levels both provoke the features of EIB including airway hyperresponsiveness, airway inflammation and remodeling (Fig. 8). Another important finding was the reversal effect of MoTr on various changes of OVA-treated rats. This could be attributed to the attenuation of NGF-induced neuron transition of AMCC. To our knowledge, this is the first study to propose the conversion of AMCC to neurons in the mechanisms of EIB, which may be helpful for identifying prospective targets for EIB intervention.

REFERENCES


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