10-1-2018

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Moderate Exercise Enhances Endothelial Progenitor Cell Exosomes Release and Function

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ABSTRACT

MA, C., J. WANG, H. LIU, Y. CHEN, X. MA, S. CHEN, Y. CHEN, J. BIHL, and Y. YANG. Moderate Exercise Enhances Endothelial Progenitor Cell Exosomes Release and Function. *Med. Sci. Sports Exerc.*, Vol. 50, No. 10, pp. 2024–2032, 2018. **Purpose**: Exercise has cardiovascular benefits which might be related to endothelial progenitor cells (EPC). Meanwhile, there is evidence suggesting that EPC-derived exosomes (EPC-EX) promote vascular repair and angiogenesis through their carried microRNA (miR)-126. In this study, we investigated whether exercise could increase the levels of circulating EPC-EX and their miR-126 cargo, and by which promote the protective function of EPC-EX on endothelial cells (EC). **Methods**: Plasma EPC-EX from sedentary, low, or moderate exercise mice, respectively, denoted as EPC-EX^S, EPC-EX^L, and EPC-EX^M, were isolated using microbead-based sorting techniques and characterized by nanoparticle tracking analysis, Western blot, and quantitative real-time polymerase chain reaction assessments of biomarkers and miR-126. High glucose (25 mM) with hypoxia (1% O_2) was used for inducing an EC injury model. The injured EC were treated by coculturing with vehicle, EPC-EX^S, EPC-EX^L, EPC-EX^M, or EPC-EX^M + anti–miR-126. After that, EC were used for flow cytometry analysis of apoptosis, assessments of tube formation and migration, and measurements of miR-126 level and its downstream sprouty-related protein-1 (SPRED1) and vascular endothelial growth factor (VEGF). **Results**: 1) Isolated EPC-EX positively expressed exosomal markers (CD63 and Tsg101) and EPC markers (CD34 and VEGFR2). 2) Exercise intensity dependently elevated plasma level of EPC, EPC-EX/EPC ratio, and miR-126 expression in EPC and EPC-EX. 3) Injured EC displayed apoptosis increment, angiogenic dysfunction and miR-126 reduction. 4) EPC-EX^M had better effects than EPC-EX^S and EPC-EX^L on alleviating those changes of injured EC, accompanied with SPRED1 downregulation and VEGF upregulation. 5) The effects of EPC-EX^M were abolished by miR-126 knockdown. **Conclusions**: Our data demonstrate that exercise can increase EPC-EX release and miR-126 level and enhance the effects of EPC-EX on protecting EC against injury through the SPRED1/VEGF pathway. **Key Words:** EXERCISE, ENDOTHELIAL PROGENITOR CELLS-DERIVED EXOSOMES, ENDOTHELIAL CELLS, microRNA-126

Exercise has beneficial impacts on cardiovascular diseases such as diabetes mellitus (1) and ischemic stroke (2). It is known that endothelial dysfunction contributes to the pathogenesis of diabetes and ischemic stroke (3). Increasing evidence suggest that exercise could act to precondition endothelial cells (EC) against shear stress injury (4) and to improve endothelial function through increasing endothelial nitric oxide synthase activity (5). However, the full molecular mechanisms of exercise-induced benefits on EC are far from fully understood.

Endothelial progenitor cells (EPC) are able to differentiate into EC for repairing endothelium injury and angiogenesis. The extent of circulating EPC pool is considered as a mirror of cardiovascular health (6). It is known that EPC are reduced in number and impaired in function in diabetes (7). Numerous studies have provided evidence showing exercise can enhance the mobilization and function of EPC (8,9). A single bout of exercise is able to improve endothelial vasodilation in chronic heart failure patients (10) and to increase circulating EPC in healthy subjects (11).

More interestingly, recent evidence suggests that exercise increases the level of total exosomes (EX) in the circulation (12), although the cellular source of EX has not been explored. Extracellular vesicles including EX and microvesicles (MV)

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C. M. and J. W. made equal contributions.
Submitted for publication December 2017.
Accepted for publication May 2018.

0195-9131/18/5010-2024/0
MEDICINE & SCIENCE IN SPORTS & EXERCISE®

DOI: 10.1249/MSS.0000000000001672

are newly recognized intercellular communicators which can mediate tissue/organ cross-talks. The functions of EX are based on the origin and status of cells which affect EX cargoes (13–15). A latest report demonstrates that obese adipose tissue macrophage-derived EX modulate insulin sensitivity in target cells through their carried microRNA (miR)-155 (15). Stem cell-derived extracellular vesicles are suggested to enact the function of stem cells (16,17). Pluripotent stem cell-derived EX have been shown to prevent cardiomyocyte apoptosis in the ischemic myocardium (17). Our group has reported that EPC-MV released under starvation or inflammation stimulation function differently on hypoxia/reoxygenation-injured EC (18). Endothelial progenitor cell-derived exosomes can promote vascular repair in balloon-induced endothelium injury model (19). Previous studies have demonstrated high glucose (HG) and hypoxia alone could result in EC apoptosis (20,21), which could mimic the *in vivo* situations of EC in diabetes with ischemic stroke insult. Whether EPC-EX from exercise mice could improve EC function against HG and hypoxia injury requires more study.

Accumulating data suggest that EX alter the function of recipient cells by transferring miR (22,23). It is well known that miR-126 modulates angiogenesis and vascular integrity. Endothelial progenitor cell microvesicle have been shown to enhance angiogenesis of human pancreatic islets and protect kidneys from ischemia/reperfusion injury through their carried miR-126 and miR-296 (24,25). Our recent work demonstrated that EPC-MV from healthy controls had beneficial effects on EPC through conveying their carried miR-126 (26). According to the miR target prediction and functional study database and Targetscan search, sprouty-related protein-1 (SPRED1) is one of direct targets of miR-126. Previous reports have also shown that miR-126 can target SPRED1/vascular endothelial growth factor (VEGF) pathway to decrease endothelial apoptosis (27,28). Notably, exercise can increase the level of miR-126 in the circulation, heart, and vascular (29–31). However, whether miR-126 expression in plasma EPC-EX can be altered by exercise remains unknown.

In this study, we aimed to investigate whether exercise can enhance the level and function of circulating EPC-EX on EC challenged by HG and hypoxia through the miR-126/SPRED1/VEGF pathway.

MATERIALS AND METHODS

Animals. C57BL/6J mice (8–10 wk of age; weight ranges from 20 to 26 g) were used for exercise experiments. Mice were maintained in a 22°C room with a 12-h light/dark cycle and fed with standard chow and drinking water *ad libitum*. All experimental procedures were approved by the Wright State University Laboratory Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health.

Treadmill exercise. Mice were randomly grouped into sedentary (no exercise), low exercise and moderate exercise groups (n = 4–6 per group). Body weights were recorded weekly. Treadmill exercise intensity was based on previous studies showing exercise has beneficial effects on cardiovascular diseases (32,33). Briefly, mice were placed on the treadmill (Columbus Instruments, Columbus, OH) and allowed to adapt to the surroundings for 3 to 5 min before starting. The treadmill speed was 5 $m \cdot min^{-1}$ for low exercise and 10 $m \cdot min^{-1}$ for moderate exercise. A daily 60-min exercise was repeated for 4 wk (5 $d \cdot wk^{-1}$).

Isolation of circulating EPC from peripheral blood. After 24 h of the last bout of exercise, blood samples were taken from the hearts of sedentary, low exercise and moderate exercise mice using 1 ml syringe with a small amount of heparin for anticoagulation. The levels of circulating EPC were determined using CD34 and VEGFR2 according to our previous studies (34). Briefly, the whole blood (800–1000 μL) was 2× diluted with filtered PBS and then gently layered over 4 ml of lymphocyte separation liquid (Sigma, St. Louis, MO) for centrifugation at 800g for 30 min at 4°C. For analysis of the levels of circulating EPC, the cells in the interface layer were incubated with 2 μL of fluorescein isothiocyanate-conjugated antimouse CD34 (eBioscience) and 5 μL of PE-conjugated antimouse VEGFR2 (BD Bioscience) antibodies for 30 min at 4°C in the dark. Isotype-matched (IgG) nonspecific antibodies were used as negative controls. Labeled EPC were analyzed using a flow cytometer (Accuri C6 Cytometers). Endothelial progenitor cell were defined as $CD34^+VEGFR2^+$ cells. The numbers of EPC were described as the $CD34^+$ $VEGFR2^+$ cells per mL of whole blood.

Isolation of circulating EPC-EX from peripheral blood using magnetic activated cell sorting. The whole blood (800–1000 μL) was 2× diluted with filtered PBS and centrifuged at 400g for 35 min at 4°C, followed by 2000g for 20 min to remove platelets. The supernatant was centrifuged at 20,000g for 70 min, and then the remaining supernatant was ultracentrifuged at 170,000g for 90 min to pellet circulating EX. The pelleted EX were resuspended with filtered PBS and then isolated by anti-CD34 and anti-VEGFR2 conjugated microbeads as previously reported by us (35) and others (36) with slight modification. In brief, the pelleted EX were incubated with 10 μL of biotin-conjugated anti-CD34 antibody (Miltenyi Biotec) in a 100-μL reaction volume for 2 h, followed by adding 10 μL of antibiotin microbeads (Miltenyi Biotec). Then, the samples were placed in a magnet (DynaMag-2; Life Technology) to separate microbeads-conjugated EX (considered as $CD34^+$ EX) from the total EX. The isolated $CD34^+$ EX were then resuspended with 100 μL filtered PBS and added with 10 μL of multisort release reagent (Miltenyi Biotec) for 10 min to cleave off the microbeads from EX by following the manufacturer's instruction. Then, all samples were placed at a magnet (DynaMag-2; Life technology) to get rid of the released microbeads overnight. On the following day, the fluid was collected and considered as $CD34^+$ EX suspension. The $CD34^+$ EX were then incubated with 10 μL of Biotin-conjugated anti-VEGFR2 antibody (Miltenyi Biotec) in a 100-μL reaction

for 2 h, followed by adding 10 μL of antibiotin microbeads (Miltenyi Biotec). Then microbeads-conjugated $CD34^+$ EX (considered as $CD34^+VEGFR2^+$ EPC-EX) were separated by the magnet. 10 μL of multisort release reagent (Miltenyi Biotec) was added to the EX suspension to cleave off the microbeads from $CD34^+VEGFR2^+$ EPC-EX. The isolated EPC-EX were analyzed by nanoparticle tracking analysis (NTA) and western blot. The EPC-EX isolated from sedentary, low exercise and moderate exercise mice were denoted as EPC-EX^S, EPC-EX^L and EPC-EX^M, respectively. The absolute number of EPC-EX was expressed as the counts of $CD34^+VEGFR2^+$ EPC-EX per ml of whole blood. The ratio of EPC-EX/EPC was calculated as: the number of EPC-EX divided by the number of EPC. The rest of EPC-EX were used for coculture experiments.

NTA of EPC-EX. The isolated EPC-EX were verified and counted by fluorescence Nanosight NS300 (Malvern Instruments, Malvern, UK) as we previously reported (35). In brief, the isolated EPC-EX were incubated with rabbit antigoat IgG conjugated with Q-dots 655 (1: 350; LifeTechnologies) in a reaction volume of 100 μL for 90 min at room temperature. The EPC-EX suspension was then added with filtered PBS to reach a final volume of 700 μL and enumerated by NTA under light scatter mode and fluorescence mode.

Cell culture. Brain EC were purchased from Cell Systems (Kirkland, WA) and cultured with CSC complete medium (Catalog 4Z0-500-R; Cell Systems) containing 10% serum, 2% human recombinant growth factors, and 0.2% antibiotic solution under standard cell culture conditions (5% CO2, 37°C). Medium was changed twice a week.

HG- and hypoxia-challenged EC model. To mimic *in vivo* diabetic and/or ischemic condition, we produced an HG and hypoxia dual injury model as we previously reported with slight modification (18). In brief, EC were divided into several groups: 1) control: cultured in CSC medium; 2) HG: challenged with 25 mM HG in CSC medium for 24 h in normoxic condition; 3) hypoxia: cultured in a normoxic condition for 18 h and then in hypoxic condition (1%, 5% CO_2, and 94% N_2; Biospherixhypoxia chamber, NY) for 6 h, followed by 24-h reoxygenation in normoxic condition; 4) HG and hypoxia: challenged with CSC complete medium containing 25 mM HG for 18 h and then subjected to 1% O_2 hypoxic culture for 6 h, followed by 24-h reoxygenation in normoxic condition. The HG- and hypoxia-challenged EC model was used for coculture experiments.

Dose–response study of EPC-EX on EC challenged by HG and hypoxia. To find the effective dose of EPC-EX on rescuing EC from HG and hypoxia injury, we cocultured HG- and hypoxia-injured EC with different concentrations of EPC-EX during the reoxygenation period: 0 (coculture medium only), 2×10^6 EPC-EX per milliliter, 20×10^6 EPC-EX per milliliter, 40×10^6 EPC-EX per milliliter, 100×10^6 EPC-EX per milliliter. Endothelial cells cultured in 12-well plates with 70% to 80% confluency were used for this experiment. After 24 h of coculture, EC were harvested for cell viability and apoptosis assays. The concentration of EPC-EX was determined by NTA.

Viability assay of EC. The viability of EC treated with various concentration of EPC-EX was determined using the MTT Assay Kit (Invitrogen, NY) as we previously reported (18). In brief, after 24 h of coculture, the cultures were replaced with 100 μL of fresh CSC culture medium. Cells in 96-well plate were added with 10 μL of 12 mM MTT solution and incubated at 37°C for 4 h. Then, 100 μL of the sodium dodecyl sulfate-HCl solution was added to each well and incubated at 37°C for 4 h. Finally, the 96-well plate was read by a microtiter plate reader (Packard) at 535 nm. The percentage of viability was defined as the relative absorbance of the cocultured cells versus the controls.

Coincubation of EPC-EX with HG- and hypoxia-challenged EC. Based on dose–response study, we chose 40×10^6 EPC-EX per milliliter as the effective concentration for the subsequent studies. During the reoxygenation period, HG and hypoxia injured EC (60%–70% confluency) were divided into five coculture groups: vehicle (coculture medium only), EPC-EX^S, EPC-EX^L, EPC-EX^M or EPC-EX^M + anti–miR-126. For knocking down the effect of miR-126, EC were transfected with miR-126 inhibitor as we previously reported with slight modification (26). In brief, in the EPC-EX^M + anti–miR-126 coculture group, miR-126 inhibitor or miR-126 scramble control (1 nM, Dharmacon) mixed with DharmaFECT transfection reagent (Dharmacon) were added to the culture medium containing EPC-EX^M. After 24 h of coculture, EC were used for various tests as described below. The transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

qRT-PCR analysis. The RNA from EPC-EX and EC were extracted using Trizol (Thermo Fisher Scientific). For determining miR-126 level, reverse transcription (RT) reactions were performed using PrimeScript^{TM} RT reagent kit (TaKaRa, Japan) and PCR reactions were conducted using SYBR Premix EX Taq^{TM} II kit (TaKaRa, Japan). The RT primer was: 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GATACG AC CGC ATT-3′. The forward primer of miR-126 was: 5′-AGG CGC TCG TAC CGT GAG TAA TA-3′. The reverse primer of miR-126 was: 5′-CCA GTG CAG GGT CCG AGG TA -3’. The expression of U6 was used as endogenous control for each sample. The forward primer of U6 was: 5′-CTCGCTTCGGCAGCACA-3′, the reverse primer of U6 was: 5′-AACGCTTCACGAATTTGCGT-3′. Relative expression level of each gene was normalized to U6 and calculated using the $2^{-\Delta\Delta CT}$ methods.

Apoptosis assay of EC. After 24 h of coculture, EC were detached for apoptosis assay by using the fluorescein isothiocyanate Annexin V apoptosis detection kit (BD Biosciences, CA) (18). The percentage of apoptotic cells was analyzed by a flow cytometer (Accuri C6, BD) and calculated as: Annexin V^+/PI^- cells/total cells $\times$ 100%.

The tube formation and migration assays of EC. The tube formation assay and migration ability of EC were conducted using *in vitro* angiogenesis assay kit and Boyden Chamber (Chemicon, Rosemont, IL) as we previously described (18;26).

Western blot analysis. The proteins of the various types of EPC-EX and EC in different coincubation groups were extracted with cell lysis buffer (Thermo Fisher scientific, Waltham, MA) supplemented with complete mini protease inhibitor tablet (Roche, Basel, Switzerland). The EC protein lysates (20 μg) and EX protein lysate (2 μg) were then electrophoresed through sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto Polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature, and incubated with primary antibody against CD63 (1: 500), Tsg 101 (1: 1000), CD34 (1: 1000), VEGFR2 (1: 1000), SPRED1 (1: 1000), VEGF (1: 1000), β-actin (1:4000; Sigma) or GAPDH (1:4000; Sigma) at 4°C overnight. All antibodies except β-actin and GAPDH were purchased from Thermo Fisher Scientific. On the next day, membranes were washed and incubated with horseradish-peroxidase–conjugated antirabbit or antimouse IgG (1:40000; Jackson Immuno Research Lab, West Grove, PA) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence developing solutions and images were quantified under ImageJ software.

Statistical analysis. Each *in vitro* experiments were repeated for four to six times. Data are expressed as the mean ± SEM. Multiple comparisons were analyzed by one- or two-way ANOVA followed by Tukey *post hoc* test. SPSS 23.0 statistical software was used for analyzing the data. For all measurements, $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of EPC-EX. The microbeads isolated EX were analyzed by NTA and Western blot analyses. As shown in Figure 1A, there were higher numbers of $CD34^+$ EX and $CD34^+VEGFR2^+$ EX isolated from the plasma of low exercise mice than that of sedentary mice. Among the three groups, moderate mice had the highest levels of $CD34^+$ EX and $CD34^+VEGFR2^+$ EX. Because EPC are double positive for CD34 and VEGFR2 expressions, the EX sorted by both CD34 and VEGFR2 conjugated microbeads were defined as EPC-EX. As shown in Figure 1B, the average diameter of the three types of EPC-EX was 110 ± 8 nm, 112 ± 5 nm, 114 ± 6 nm, respectively, indicating different intensity of exercise did not significantly change the sizes of EPC-EX. According to the Western blots results, the isolated EPC-EX expressed exosomal markers CD63 and Tsg 101, as well as EPC markers CD34 and VEGFR2. These data demonstrate that the EPC-EX were successfully isolated from the plasma samples.

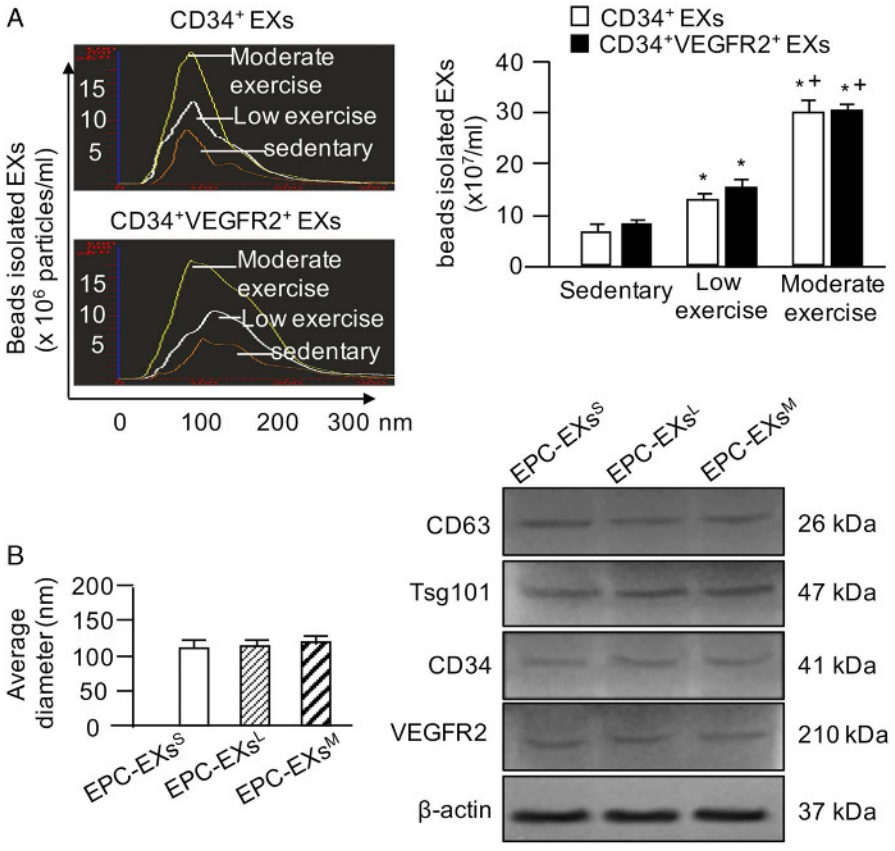

FIGURE 1—Characterization of the isolated EPC-EX from sedentary, low and moderate exercise mice by fluorescence NTA and western blot analyses. A, Representative NTA plots of $CD34^+$ EX and $CD34^+VEGFR2^+$ EX in the three groups and summarized data showing the concentration of beads isolated EX. B, Summary data showing the diameter and the Western blot bands showing the expressions of CD63, Tsg 101, CD34, VEGFR2, and β-actin of the three types of EPC-EX. *$P < 0.05$, vs sedentary; +$P < 0.05$, vs low exercise. Data are expressed as mean ± SEM, n = 4/group.

TABLE 1. Analysis of the levels of EPC, EPC-EX and miR-126.

Parameters	Sedentary	Low Exercise (5 m·min^{-1})	Moderate Exercise (10 m·min^{-1})
Body weight (g)	24.6 ± 0.24	23.7 ± 0.15	23.1 ± 0.43
EPC (number per mL)	497 ± 10	534 ± 10*	589 ± 14*,**
EPC-EX ($\times 10^7$ n·mL^{-1})	2.2 ± 0.3	4.2 ± 0.4*	9.5 ± 0.6*,**
EPC-EX/EPC ($\times 10^3$)	44 ± 5	78 ± 6*	161 ± 8*,**
MiR-126 level in EPC (fold)	1 ± 0.02	1.25 ± 0.05*	1.55 ± 0.03*,**
MiR-126 level in EPC-EX (fold)	1 ± 0.04	1.5 ± 0.04*	2.3 ± 0.05*,**
MiR-126 level in EPC-EX/EPC	1.4 ± 0.03	2.0 ± 0.05	2.8 ± 0.07

Moderate exercise was more effective in increasing the numbers of EPC and EPC-EX and their miR-126 expression.
Data are expressed as mean ± SEM, n = 6/group.
*$P < 0.05$, vs sedentary.
**$P < 0.05$, vs low exercise.

Moderate exercise had prominent effects on increasing the level of EPC and the release of EPC-EX as well as their carried miR-126. As shown in Table 1, after 4-wk exercise training, there was no significant difference change of the body weight of mice among the three groups. As compared with sedentary mice, the level of EPC was significantly elevated by low exercise, which was further raised by moderate exercise. Meanwhile, our data showed that the release ratio of EPC-EX was higher in moderate mice than that of low exercise or sedentary mice. As revealed by qRT-PCR analysis, low exercise upregulated miR-126 expression in EPC and their released EX as compared with that of sedentary mice. Moreover, moderate exercise further increased miR-126 level in EPC and their relative EPC-EX. Meanwhile, our data showed that miR-126 was enriched in EPC-EX as compared with parent EPC. These data indicate that exercise could increase the numbers and the miR-126 levels in EPC and EPC-EX in an intensity-dependent manner.

HG- and hypoxia-induced apoptosis and dysfunction of EC which were associated with downregulation of miR-126 expression. As shown in Figure 2, HG or hypoxia alone increased the apoptosis of EC as compared with that of normoxic cultured EC, whereas combination of HG and hypoxia further promoted EC to enter into early apoptotic status. Similarly, the tube formation and migration abilities of EC were compromised by HG or hypoxia alone as revealed by less tube formed in each field and less EC pass through the semipermeable membrane of the Boyden chamber. Combination of HG and hypoxia further worsened the angiogenic abilities of EC. Of note, our data revealed that miR-126 level was decreased in EC exposed to HG or hypoxia, which was further decreased in EC subjected to HG and hypoxia. These data suggest that the success of the EC injury model and the injury of EC is associated with the downregulation of miR-126.

EPC-EX dose-dependently increased cell survival in EC challenged by HG and hypoxia. To find the effective dose of EPC-EX on rescuing EC from HG- and hypoxia-induced injury, we performed dose–response experiments. Our data (Fig. 3) showed that a dose of 2×10^6 EPC-EX per milliliter did not, whereas, 20×10^6 EPC-EX per milliliter changed the proliferation ability and apoptosis rate of EC challenged by HG and hypoxia. These effects were more seen at doses of 40×10^6 EPC-EX per milliliter and 100×10^6 EPC-EX per milliliter. Therefore, we chose the medium dose (40×10^6 EPC-EX per milliliter) for coincubation functional experiments.

EPC-EX^M had better efficiencies on decreasing the apoptosis of EC challenged by HG and hypoxia via upregulating miR-126 level. After coincubation with the different types of EPC-EX, we analyzed the apoptosis of EC. Our results (Fig. 4A) showed that EPC-EX^S significantly decreased HG- and hypoxia-induced EC apoptosis as compared with that of vehicle. EPC-EX^L exhibited a better effect on decreasing EC apoptosis than EPC-EX^S. Furthermore, EPC-EX^M exhibited the best effects among the three types of EPC-EX.

To further clarify whether miR-126 is the key components in the antiapoptotic effect of EPC-EX, we determined the level of miR-126 in EC. According to the qRT-PCR analysis, we found (Fig. 4B) that coincubation of EPC-EX^S or EPC-EX^L upregulated miR-126 level in EC which was further increased by EPC-EX^M. Transfection of miR-126 inhibitors effectively decreased the upregulation of miR-126 elicited by EPC-EX^M incubation and blocked the antiapoptotic effect elicited by EPC-EX^M. All together, these data indicate that EPC-EX have antiapoptotic effect on HG- and hypoxia-challenged EC through upregulating miR-126 level.

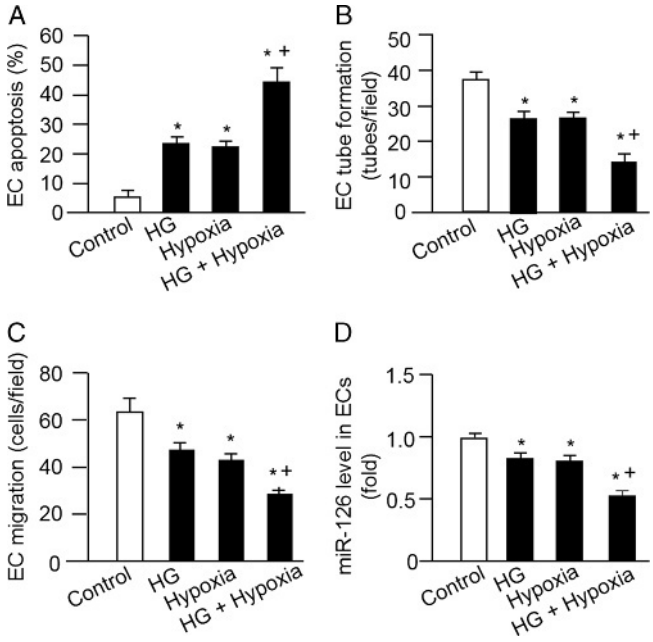

FIGURE 2—Combination of HG and hypoxia increased EC apoptosis and dysfunction associating with the downregulation of miR-126 expression. A, EC apoptosis; B, EC tube formation; C, EC migration; D, miR-126 level in EC. *$P < 0.05$, vs control. +$P < 0.05$, vs HG or hypoxia. Data are expressed as mean ± SEM, n = 4 per group.

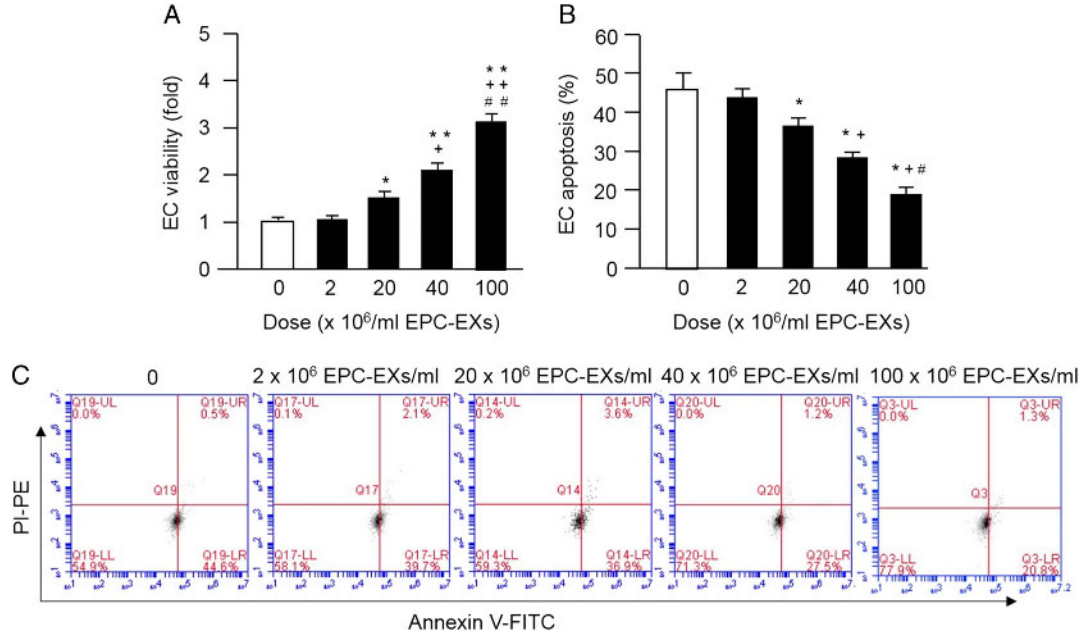

FIGURE 3—Incubation of EPC-EX dose-dependently increased cell survival in the EC injury model challenged by HG and hypoxia. A, EC viability; B, EC apoptosis; C, representative flow cytometry plots of EC. *$P < 0.05$, vs 0 or 2×10^6 EPC-EX per milliliter; **$P < 0.01$, vs 2×10^6 EPC-EX per milliliter; +$P < 0.05$, vs 20×10^6 EPC-EX per milliliter; ++$P < 0.05$, vs 20×10^6 EPC-EX per milliliter; **$P < 0.05$, vs 40×10^6 EPC-EX per milliliter; Data are expressed as mean ± SEM, $n = 4–6$ per group.

EPC-EX^M ameliorated the angiogenic functions of EC challenged by HG and hypoxia through a miR-126 dependent mechanism. As shown in Figures 4C-D, the tube formation and migration ability of HG- and hypoxia-challenged EC were significantly improved by EPC-EX^S or EPC-EX^L coincubation as compared to that of vehicle (culture medium only). What is more, EPC-EX^M further improved the angiogenic abilities of EC, which was blocked by miR-126 inhibitor. These data suggest that EPC-EX have proangiogenic effects on EC which depend on miR-126 expression.

EPC-EX–modulated SPRED1 and VEGF expressions of EC challenged by HG and hypoxia through a miR-126–dependent mechanism. To further explore

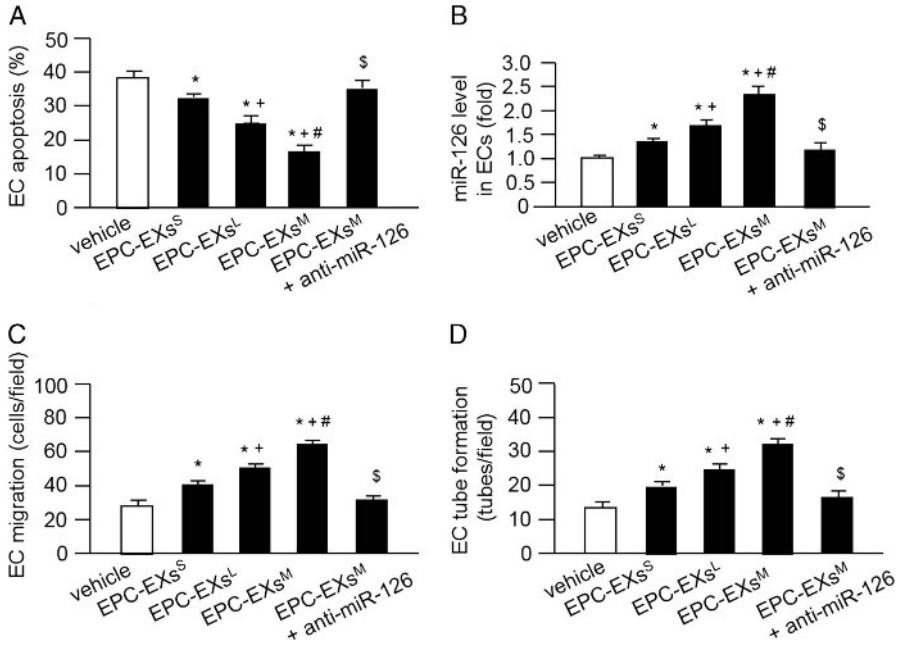

FIGURE 4—Incubation of EPC-EX isolated from moderate exercise mice had better efficiencies on decreasing the apoptosis and improving the angiogenic functions of EC challenged by HG and hypoxia via upregulating miR-126 level. A, apoptosis rate of EC; B, miR-126 level in EC; C, migration ability of EC; D, tube formation ability of EC; *$P < 0.05$, vs vehicle; +$P < 0.05$, vs EPC-EX^S; #$P < 0.05$, vs EPC-EX^L; \$$P < 0.05$, vs EPC-EX^M. Data are expressed as mean ± SEM, $n = 4–6$ per group.

the mechanisms of EPC-EX, we determined the expression of miR-126 target molecule SPRED1. As shown in Figure 5, EPC-EX^S and EPC-EX^L alone coincubation decreased SPRED1 protein expression which was further decreased by EPC-EX^M. It is known that SPRED1 can modulate VEGF expression. We found that VEGF expression was upregulated in EC coincubated with EPC-EX^S or EPC-EX^L which was further elevated by EPC-EX^M. Knockdown of miR-126 significantly blocked the changes of SPRED1 and VEGF.

DISCUSSION

In this study, we explored the regulatory effects of exercise on circulating EPC-EX level and miR-126 cargo. Their protective function was tested on EC challenged with HG and hypoxia. We also investigated the relevant mechanism of the protective effects of EPC-EX by focusing on miR-126 downstream proteins SPRED1 and VEGF. There are several major findings: 1) intensity-dependent exercise for 4 wk increased the level of circulating EPC-EX and their miR-126 cargo; 2) EPC-EX isolated from moderate exercise mice had prominent effects on protecting EC against HG- and hypoxia-induced apoptosis and angiogenic dysfunction; 3) These effects elicited by EPC-EX were associated with the modulation of miR-126/SPRED1/VEGF.

Previous evidence suggests that a single bout exercise can increase the number and improve the function of EPC in healthy individuals (11). Five weeks of moderate exercise training has been shown to produce significant cardiovascular benefits by improving mitochondrial function and 10 wk of moderate exercise can abates endothelial dysfunction in the aorta in db/db mice (32,33). Here, we investigated whether low- and moderate-intensity exercise for 4 wk could affect the level of circulating EPC in healthy mice. Our data showed that 4 wk of low- and moderate-intensity exercise respectively increased the number of EPC in the plasma by 7% and 18%, suggesting an intensity-dependent effect of exercise. This finding is consistent with previous evidence showing that exercise can enhance the mobilization of EPC from bone marrow. More interestingly, we found that miR-126 level was increased in the circulating EPC isolated from both low and moderate exercise. This is partially supported by previous reports showing that exercise can increase miR-126 level in skeletal muscle and vessels which contributes to vascular network homeostasis (31,37).

It is well known that EX can be released from a variety type of cells and their functions may be determined by the cell origin and cellular status (14). Recent evidence suggest that exercise can increase the level of total circulating EX (12), but the subpopulations of EX have not been explored. To identify whether exercise can modulate the level and cargo of circulating EPC-EX, we isolated EPC-EX from different intensity of exercise mice by using a two-step microbeads activated cell sorting technique based on our previously established methods (34). First, we isolated CD34$^+$ EX from the plasma by using the antibody conjugated microbeads. We found that low exercise increased the levels of CD34$^+$ EX which were further raised by moderate exercise. Since EPC-EX coexpress CD34 and VEGFR2, and CD34 is a marker widely expressed in hematopoietic precursor cells, we sorted CD34$^+$VEGFR2$^+$ EX (defined as EPC-EX) from the population of circulating CD34$^+$ EX. Furthermore, we confirmed the isolated EPC-EX positively expressed exosomal markers (CD63 and Tsg 101) and EPC markers (CD34 and VEGFR2), and have sizes of less than 120 nm. All these characters of EPC-EX meet the guidelines of minimal extracellular vesicle updated on 2017 (38), suggesting the successful isolation of EPC-EX. What is more, we found that the number of EPC-EX and the release ratio of EPC-EX/EPC were intensity-dependently elevated by 4-wk exercise. These data demonstrate that exercise can modulate the level of circulating EPC-EX, which is consistent

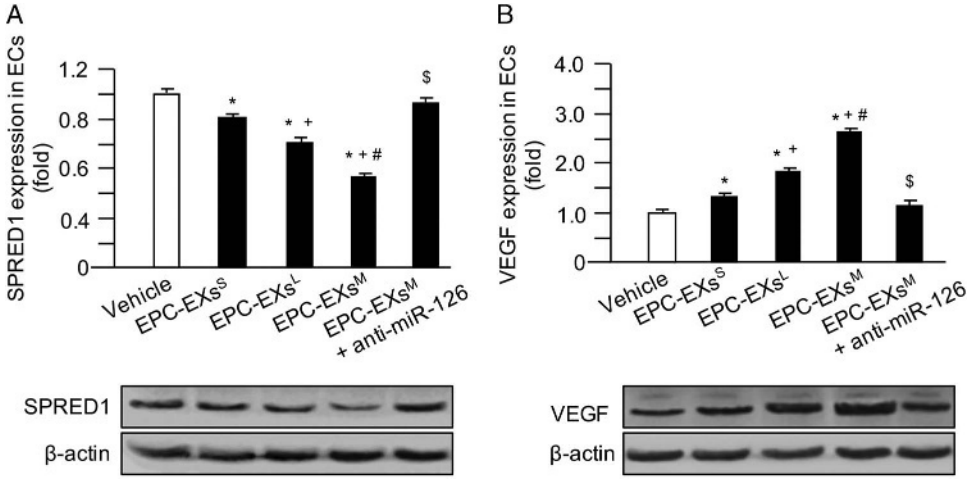

FIGURE 5—Incubation of EPC-EX isolated from moderate exercise mice had better efficiencies on downregulating SPRED1 expression and upregulating VEGF expression of EC challenged by HG and hypoxia. A, SPRED1 expression of EC; B, VEGF expression of EC. *$P < 0.05$, vs vehicle; +$P < 0.05$, vs EPC-EX^S; #$P < 0.05$, vs EPC-EX^L; \$$P < 0.05$, vs EPC-EX^M. Data are expressed as mean ± SEM, $n = 4$ per group.

with a previous study demonstrating that exercise can increase the total circulating level of EX (12). Of note, we found that miR-126 was enriched in EPC-EX from sedentary mice. What is more, the miR-126 level in EPC-EX was raised by exercise in an intensity-dependent manner as seen in their parent EPC, indicating that EPC-EX carry the molecular signature of EPC.

A large body of evidence indicate that the functions of EX can be affected by their cargoes (13–15). In this study, we investigated whether exercise-induced elevation of miR-126 expression in EPC-EX is associated with the function of EPC-EX on EC. Previous studies have demonstrated HG and hypoxia alone could lead to increased EC apoptosis and dysfunction (20,21). Here, we induced an EC injury model by dual challenge of HG and hypoxia which mimic the *in vivo* situation in diabetes with ischemic stroke attack. We found that this model of EC injury had decreased survival ability and compromised angiogenic capacity, which was associated with miR-126 downregulation. Then, we conducted dose–response experiments to determine the treatment effects of EPC-EX on the injured EC model. We found that EPC-EX from sedentary mice could dose-dependently increase the viability and decrease the apoptosis of EC, suggesting EPC-EX plays a protective role in EC against HG and hypoxia injury. To further clarify whether exercise could modulate the protective effects of EPC-EX, we treated HG- and hypoxia-injured EC with the EPC-EX isolated from different exercise intensity mice. Our data showed that the EPC-EX from low exercise mice exhibited antiapoptotic and pro-angiogenic effects on EC. These effects were more seen in the EPC-EX isolated from mice under higher-intensity exercise, suggesting that exercise modulate the protective effects of EPC-EX on EC injury. As discussed above, EPC-EX carry abundant miR-126. We found that miR-126 expression in EC was elevated by EPC-EX from sedentary and low exercise mice, which was even higher in EC treated by EPC-EX isolated from moderate-intensity exercise mice. These findings reflect that EPC-EX could delivery miR-126 into the target EC. This finding is supported by previous studies from other group and our laboratory demonstrating that EPC-derived extracellular vesicles including EX carry miR-126 (18,25). To further clarify the role of miR-126 in the protection effects of EPC-EX isolated from moderate exercise mice, we transfected the target EC with miR-126 inhibitor. Manipulating cells with specific miR mimic or inhibitor is a well-recognized approach to alter the function of a specific miR in cells and their relative EX (26,39). As expected, results showed that knockdown of miR-126 by using miR-126 inhibitor abolished the protective effects elicited by EPC-EX from moderate exercise mice. Besides, we could add miR-126 mimic to EPC-EX isolated from sedentary mice to elucidate the role of miR-126 in modulating the protective effects of EPC-EX on EC subject to HG and hypoxic injury. Altogether, these findings indicated that the antiapoptotic and proangiogenic effects of EPC-EX isolated from moderate exercise mice were ascribed to miR-126.

As an important angiogenic growth factor, VEGF has an antiapoptotic effect that prevents EC from death. Several findings have indicated that VEGF could be regulated by exercise (2,37), but its relationship with EPC-EX in exercise has not been revealed. Previous evidence has documented that the VEGF signaling pathway can be negatively regulated by SPRED1 which is one of the main targets of miR-126 (39). In this study, we found that EPC-EX isolated from sedentary and low exercise mice significantly downregulated the expression of SPRED1 which was associated with an increased VEGF level. What is more, EPC-EX from moderate exercise mice exhibited prominent effects than those isolated from low exercise mice, whereas knockdown of miR-126 remarkably blocked the abovementioned effects. These findings altogether suggest that miR-126 carried by EPC-EX can regulate the expressions of SPRED1 and VEGF in EC.

In conclusion, exercise intensity-dependently increases the release of circulating EPC-EX and miR-126 cargo, as well as regulate the protective effects of EPC-EX on EC by targeting the miR-126 downstream SPRED1/VEGF pathway. These findings help to understand the molecular mechanism of exercise-induced benefits on EC in diabetes with ischemic stroke.

There are some limitations in this study, 1) we only investigated the protective effects of mice EPC-EX on human EC. Isolating EPC-EX from human subjects undergoing different exercise intensities and investigate their roles in human EC need to be performed in future studies. 2) We have not excluded the possible interactions of endogenous EX in the media and exogenously added EX. Such interaction might also affect the function of HG- and hypoxia-injured EC. 3) Because the plasma EPC-EX were used for coculture experiments, we could not directly manipulate EX miR-126 level. Instead, we blocked miR-126 expression in the target cells (EC) to clarify the role of miR-126.

This work is supported by the National Natural Science Foundation of China (30700388), the Key Program of Natural Science Foundation of Hubei Province, China (2015CFA084), the Outstanding Young Scientific and Technological Innovation Team in the Colleges and Universities of Hubei Province, China (T201523), the Scientific Research Project supported by Wuhan Sports University (2014QZ07, 2016XH24) and American Diabetes Association (1-17-IBS-187). The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation, and do not constitute endorsement by ACSM.

There are no conflicts of interests.

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