Physical Training Increases Endothelial Progenitor Cells, Inhibits Neointima Formation, and Enhances Angiogenesis

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Background—The molecular mechanisms by which physical training improves peripheral and coronary artery disease are poorly understood. Bone marrow–derived endothelial progenitor cells (EPCs) are thought to exert beneficial effects on atherosclerosis, angiogenesis, and vascular repair.

Methods and Results—To study the effect of physical activity on the bone marrow, EPCs were quantified by fluorescence-activated cell sorter analysis in mice randomized to running wheels (5.1 ± 0.8 km/d, n = 12 to 16 per group) or no running wheel. Numbers of EPCs circulating in the peripheral blood of trained mice were enhanced to 267 ± 19%, 289 ± 22%, and 280 ± 25% of control levels after 7, 14, and 28 days, respectively, accompanied by a similar increase of EPCs in the bone marrow and EPCs expanded from spleen-derived mononuclear cells. eNOS−/− mice and wild-type mice treated with Nω-nitro-L-arginine methyl ester showed lower EPC numbers at baseline and a significantly attenuated increase of EPC in response to physical activity. Exercise NO dependently increased serum levels of vascular endothelial growth factor and reduced the rate of apoptosis in spleen-derived EPCs. Running inhibited neointima formation after carotid artery injury by 22 ± 2%. Neoangiogenesis, as assessed in a subcutaneous disc model, was increased by 41 ± 16% compared with controls. In patients with stable coronary artery disease (n = 19), moderate exercise training for 28 days led to a significant increase in circulating EPCs and reduced EPC apoptosis.

Conclusions—Physical activity increases the production and circulating numbers of EPCs via a partially NO-dependent, antiapoptotic effect that could potentially underlie exercise-related beneficial effects on cardiovascular diseases. (Circulation. 2004;109:220-226.)

Key Words: exercise ■ cells ■ nitric oxide ■ angiogenesis ■ coronary disease

Regular physical activity is associated with a decrease in the incidence of cardiovascular events.1-2 Physical training improves endothelial function, exercise capacity, and collateralization in patients with coronary artery disease,3,4 chronic heart failure,5,6 and peripheral artery disease.7,8 Physical activity is associated with improved mood, body weight, blood pressure, insulin sensitivity, and hemostatic and inflammatory variables.9,10 However, despite the wealth of evidence derived from epidemiological and interventional trials, there is limited understanding of the underlying molecular mechanisms. Notably, physical training reduces vascular oxidative stress at least in part via increased activity of endothelial nitric oxide synthase (eNOS) and extracellular superoxide dismutase, which in turn could exert beneficial vascular effects.11,12

Recent evidence has shown that vascular function not only depends on cells that reside within the vessel wall but also appears to be significantly modulated by circulating cells derived from the bone marrow.13 A specific subset of these stem cells has been shown to enhance angiogenesis, promote vascular repair, improve endothelial function, inhibit atherosclerosis, and increase ventricular function after myocardial infarction.14-19 This circulating bone marrow–derived cell population has been named endothelial progenitor cells (EPCs), characterized by coexpression of Sca-1 and vascular endothelial growth factor receptor 2 (VEGFR2).14,17,19,20 We refer to these cells as EPCs throughout this report. Numbers of EPCs are regulated. Although myocardial infarction and bypass surgery acutely increase the circulating numbers, accumulation of vascular risk factors appears to reduce these bone marrow–derived cells, which suggests that vascular health and repair processes after injury require increased numbers of this potentially beneficial population of cells.17,20-23 Therefore, we reasoned that physical activity may influence the numbers of EPCs and tested this hypothesis in mice subjected to a physical active lifestyle versus a
Methods

Animals and Exercising
Animal experiments were conducted in accordance with institutional guidelines. Male C57Bl/6 (Charles River Laboratories, Sulzfeld, Germany), 129/SV (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany), and eNOS−/− (B6129/P2-No3, Charles River) mice were kept under usual care at 1 to 6 mice per cage. Each exercising mouse was kept in an individual cage supplied with a running wheel (12.8-cm diameter) equipped with a tachometer (Sigma Sport BC-401) to record the daily running distance. Mice ran voluntarily. The mean running distance was $5100 \pm 800$ m per 24 hours and did not differ in mice after carotid artery injury or disc implantation or in eNOS−/− mice. For comparison, treadmill training was performed (treadmill EXER-8, Columbus Instruments) for 3 weeks 5 times per week for 30 minutes at 12 m/min as described previously.11,12 Some C57Bl/6 mice were treated with rosuvastatin 2 mg/kg SC for 7 days. For indicated mice, N4-nitro-L-arginine methyl ester (L-NAME; Sigma) was added to the drinking water (daily dose, $\sim50$ mg/kg; concentration in drinking water, 1.5 mg/mL).24

Humans and Training
Patients with clinically stable coronary artery disease documented by angiography (n=19) underwent a 4-week controlled, standardized exercise program of bicycle ergometer training, moderate muscle strength training (lower-limb muscle training by shuttle device), and walking as described in detail previously.25 The maximum workload of ergometer training was defined to correspond to the heart rate measured at 60% to 80% of peak oxygen uptake. An initial warmup of 5 minutes at 15 W was followed by training units of 15 to 20 minutes. Patients with concomitant inflammatory disease, defined as clinical symptoms of infection or C-reactive protein $>10$ U/L, were excluded, as were patients unable to fully participate in the program for physical or mental reasons. Medication was not changed during the study period. Exercise capacity before and after the training program was assessed by peak oxygen uptake with a ramp protocol (initial level 25 W, 10-W increase every 10 minutes; Ergoline 900, Marquette/Hellige; Ergoscope, Ganshorn) and by a standardized 6-minute walking test.25,26

Fluorescence-Activated Cell Sorter Analysis
Mouse blood and bone marrow were analyzed as described previously.18,19,23 The viable lymphocyte population was analyzed for Sca-1/VEGFR2-positive cells with the corresponding phycoerythrin-labeled secondary antibody (Sigma). Isotype-identical antibodies served as controls in every experiment (Becton Dickinson). Human blood samples were processed with VEGFR (Fik-1; sc6251, Santa Cruz), anti-mouse phycoerythrin (phycoerythrin C, Sigma P-9670), and CD34 FITC (Becton Dickinson 34 58 01).19

Cultivation of Spleen-Derived EPCs
In mice, the spleen functions as a hematopoietic organ. Spleen mononuclear cells were isolated and cultured in fibronectin (Sigma) as described previously.18,19,23 After 7 days in culture, EPCs were identified by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated LDL (DiLDL, 2.4 µg/mL; CellSystems) and staining with FITC-labeled *Giffonia* (*bundeirea*) *simplicifolia* lectin I (lectin, 10 µg/mL; Vector Laboratories). Apoptotic cell death was quantified by ELISA of cytoplasmic histone-associated DNA fragments (Cell Death Detection ELISA-plus, Roche).

Isolation and Culture of Circulating Human EPCs
Mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrom) from peripheral blood and cultured on fibronectin-coated dishes in endothelial basal medium (CellSystems) supplemented with endothelial growth medium SingleQuots and 20% fetal calf serum. After 4 days in culture, adherent cells were incubated with DiLDL and stained with FITC-labeled *Ulex europaeus* agglutinin I (lectin, 10 µg/mL; Sigma).

VEGF and Stromal Cell–Derived Factor 1 Serum Levels
VEGF and stromal cell–derived factor 1 (SDF-1) serum levels were determined by ELISA (VEGF ELISA kit mouse, Oncogene Research Products; Quantikine mouse SDF-1 immunoassay, R&D Systems).

Real-Time Reverse Transcription–Polymerase Chain Reaction
Real-time reverse transcription–polymerase chain reaction was performed with the Prism 7700 Sequence Detection System, PE Biosystems. Primers for eNOS were 5′-TTCCGGCTGGCACCCT-GATCTTCA-3′ and 5′-AAATATGTCCCTGCTCAAGGGA-3′. For 18S, the primers were 5′-TTGATTAAGTCCCTGCTTTTG-3′ and 5′-CGATCCGAGGGCCTA ACTA.

eNOS Activity Assay
Conversion of [3H]arginine to [3H]citrulline was measured with the NOS assay kit from Calbiochem. Rat cerebellum served as positive control. Lysates incubated with L-NAME (1 mmol/L) served as blanks.

Carotid Injury Model
Carotid artery injury was induced as described in detail previously.11,12 Carotid arteries were harvested 14 days after surgery. Complete reendothelialization was confirmed by staining for von Willebrand factor (clone A 0082, Dako) and 4′,6-diamidino-2-phenylindole (Dapi, Linaris). Isotype-specific antibodies (Santa Cruz) were used for negative controls. For morphometric analyses, Lucia Measurement version 4.6 software was used to measure neointimal area of 25 sections per animal.

Disc Angiogenesis Model
A disc of polyvinyl alcohol sponge (Rippey), covered with nitrocellulose cell-impermeable filters (Millipore), allowed capillaries to grow only through the rim of the disc.27 The discs were implanted subcutaneously. After 14 days, space-filling fluorescent microspheres (0.2 µm; Molecular Probes) were injected into the left ventricle to deliver them to the systemic microvasculature. The area of the disc invested by fibrovascular growth was assessed with Lucia Measurement version 4.6 software.

Statistical Analysis
Results are presented as mean ±SEM. Paired and unpaired Student t tests and ANOVA for multiple comparisons were used where applicable. Post hoc comparisons were performed using the Neuman-Keuls test. Values of P<0.05 were considered significant.

Results
Physical Activity Increased EPCs in Mice
The time-dependent effect of voluntary running on the numbers of Sca-1/VEGFR2-positive cells is depicted in Figure 1. One day of running did not cause a significant increase in EPCs. After 7 days, numbers of EPCs were significantly increased in the peripheral blood, bone marrow, and spleen. This effect was sustained for up to 28 days of continuous activity.
Voluntary running of 129/SV mice resulted in similar upregulation compared with C57/Bl6 mice. To exclude a potential unspecific effect of the voluntary running in wheels, mice were subjected to forced running on a treadmill (5 times per week for 30 minutes at 12 m/min). Treadmill training for 28 days increased EPC numbers to 247/11006 23%, 160/11006 14%, and 151/11006 14% of baseline in blood, bone marrow, and spleen, respectively.

Regulation of EPCs by Endothelial Nitric Oxide
Exercise increases nitric oxide bioavailability. Figure 2A exhibits the time-dependent increase of eNOS mRNA expression in our experimental model. eNOS mRNA and protein expression and upregulation by exercise did not differ significantly between the aorta and the carotid arteries (data not shown). Voluntary exercise (28 days) increased NOS activity as assessed by [3 H]arginine to [ 3 H]citrulline conversion as assays to 329/11006 78% of control levels in the aortas and to 385/11006 89% in the carotid arteries (n=4, P<0.05). To explore the effect of endothelial NO on EPC numbers, running wheel experiments were repeated in eNOS knockout mice and wild-type mice treated with the NOS inhibitor L-NAME.

Figure 1. Training and EPC levels in mice. After 1, 7, 14, and 28 days, Sca-1/VEGFR2-positive cells were quantified by fluorescence-activated cell sorter analyses in samples of (A) peripheral blood and (B) bone marrow in trained (open bars) and untrained (solid bars) mice. C, Mononuclear cells derived from spleen homogenates were isolated and cultured for 7 days under EPC-specific conditions. EPC numbers were assessed after staining with DiLDL and lectin. Mean±SEM, n=12 to 16. *P<0.05.

Figure 2. Role of NO in exercise-induced EPC regulation. A, eNOS mRNA expression in aortic segments on day 0, 1, 7, 14, and 28 of training. Mean±SEM, n=8; *P<0.05. Effect of 28 days of exercise on EPCs in peripheral blood (B), bone marrow (C) and spleen (D) in wild-type mice, mice treated with L-NAME (50 mg · kg⁻¹ · d⁻¹, 28 days), and eNOS⁻/⁻ mice. Mean±SEM, n=8; *P<0.05 vs untrained wild type, #P<0.05 vs untrained eNOS⁻/⁻.
Exercise Increases VEGF and Inhibits EPC Apoptosis

VEGF and SDF-1 have been suggested as mediators of EPC regulation. Compared with sedentary mice, physical activity increased VEGF serum concentration (Figure 3A) after 7 and 28 days of physical activity. The increase in VEGF after day 7 was abolished by cotreatment with L-NAME. SDF-1 levels remained unchanged (Figure 3B). Circulating numbers of EPCs have been shown to depend at least in part on the rate of apoptosis. Therefore, the rate of apoptosis was quantified in EPCs expanded in vitro from wild-type mice. Figure 3C demonstrates that physical activity decreased EPC apoptosis to 54±11% of control levels.

Physical Activity Reduced Neointima Formation and Enhanced Neoangiogenesis

Neointima formation after carotid injury is dependent on the rate of reendothelialization, which is enhanced by circulating EPCs. Seven days after initiation of exercising, vascular injury was induced. Fourteen days after lesion induction, vessels were harvested and analyzed histologically. Figure 4 shows representative examples of neointima formation (A) and the quantitative histomorphometric measurement (C). Physical exercising markedly reduced the induction of neointima formation.

EPCs have been reported to significantly contribute to new blood vessel formation. Therefore, we determined whether exercise modulates the angiogenic response in vivo in a model of inflammation-induced neoangiogenesis in mice (Figures 4B and 4C). Subcutaneous implantation of a polyvinyl sponge for 14 days resulted in ingrowth of new vessels. Exercising, initiated after implantation of the disc, increased the area of neoangiogenesis by 41±16% compared with sedentary mice.

Exercise Training Increased EPC Levels in Humans

Patients with documented stable coronary artery disease (n=19) underwent a controlled, standardized exercise program of bicycle ergometer training, moderate muscle strength training, and walking for 28 days. Patient characteristics are summarized in the Table. Medications were not changed
during the training period. The exercise program increased peak VO\textsubscript{2} from 9.58 ± 4.0 to 11.8 ± 5.80 kg/mL (P < 0.05) and the 6-minute walking distance from 357 ± 98 to 422 ± 112 m (P < 0.05). Patients showed no signs of myocardial ischemia during exercise testing before and after the training period.

EPC numbers in the peripheral blood were assessed via fluorescence-activated cell sorter analysis (CD34\textsuperscript{+}/KDR\textsuperscript{+} cells) after 28 days. Figure 5A indicates a significant increase in circulating EPCs in response to training. In addition, EPCs were expanded from human blood in vitro before and after 28 days of training and identified by DiLDL uptake and lectin staining (Figure 5B). EPC numbers rose after physical training in humans (78 ± 34% increase on day 28 compared with day 1 of training). The rate of EPC apoptosis after the training program was reduced by 41 ± 11% compared with apoptosis before exercising.

**Discussion**

The present study produced several findings. (1) Physical exercise increases the numbers of EPCs in bone marrow, peripheral blood, and spleen in mice. (2) Upregulation of EPCs by exercise is dependent at least in part on endothelial NO and VEGF, and (3) exercise decreases the rate of EPC apoptosis. (4) Running diminished neointima formation after vascular injury and (5) enhanced neangiogenesis. Furthermore, (6) a 28-day exercise program upregulated circulating EPCs in patients with coronary artery disease.

Coronary heart disease evolves depending on the accumulation of risk factors that exert a wide array of molecular and cellular abnormalities within the vessel wall, which leads to the development of atherosclerotic lesions. However, the concept of local pathological processes within the vessel wall has recently been challenged by discoveries related to stem cell biology. In this context, EPCs, which resemble premature, circulating, bone marrow–derived cells with potential to transdifferentiate,\textsuperscript{13,15} depend on the occurrence of classic risk factors. Increasing numbers of risk factors lead to decreased numbers of circulating EPCs in patients with stable coronary heart disease.\textsuperscript{30,31} Given the fact that EPCs have been implicated in revascularization, vascular repair, and myocardial regeneration, it is presumable that EPC counts correlate inversely with the risk of cardiovascular diseases.\textsuperscript{13–15,20,23} However, it remains uncertain whether the Sca-1/VEGFR2-positive cell population named EPC represents a homogenous cell population. In addition, evidence is accumulating that EPC differentiation is not restricted to the endothelial lineage, but that these cells may develop into multiple cell types, eg, cardiomyocytes.

Intervention studies have demonstrated that physical training improves the outcome of patients with coronary heart disease and heart failure.\textsuperscript{1–8} The underlying mechanisms may
relate to increased shear stress on the endothelial monolayer, which in turn enhances the activity of enzymes such as eNOS and extracellular superoxide dismutase, shifting the reactive oxygen balance toward the beneficial NO.11,12 However, parallel or subsequent events are less clear. Importantly, the effect of physical exercising on EPCs has not been defined.

According to the present findings, physical activity leads to an increased number of circulating EPCs in mice and humans. This effect occurs rapidly and is sustained for at least 4 weeks. The increased circulating EPC numbers are associated with increased production within the bone marrow, because EPC numbers rise concomitantly in this compartment. Mice supplied with running wheels showed upregulation of VEGF serum levels. VEGF activates PI-3-kinase and increases EPC numbers14 and therefore may significantly contribute to the effects of exercise. Physical exercise increases NO bioavailability,12 and in concert with this fact, the effect of physical activity on EPCs is markedly reduced after inhibition or deletion of eNOS, which suggests an NO-dependent increase of EPCs in response to exercising. Statin treatment has been suggested to upregulate EPCs, potentially by an NO-mediated pathway.21 Supporting the importance of NO for EPC regulation, cotreatment with L-NAME completely inhibited the statin effect on EPCs. Concomitant treatment with an NO inhibitor abrogated the observed increase in VEGF plasma levels, which indicates that VEGF is regulated during exercise by NO. Because the effect on the bone marrow–derived cells was not completely omitted, additional mechanisms unrelated to NO are likely but remain unknown. Absolute numbers of EPCs in untrained eNOS−/− mice and mice treated with L-NAME were significantly reduced, which suggests an important role of eNOS for basal EPC regulation. In addition to an upregulation of EPC generation, physical exercise may increase EPC numbers by prolonging their lifespan. The data derived from cultured EPCs indicate that the observed EPC enhancement in the circulation and the bone marrow could be explained at least in part by antiapoptotic effects of physical activity on EPC and potentially their progenies.

EPCs are increased by ischemia, eg, in animal models of hindlimb ischemia.13 The herein-used mouse models show an NO-dependent EPC increase that is probably unrelated to oxygen supply, because ischemia is unlikely to occur in this animal model of voluntary exercise. This is supported by epidemiological data showing that exercising not only prevents recurrent vascular events but is also effective in primary prevention.1,2

To test whether voluntary exercising versus a sedentary lifestyle improves vascular physiology in mice, we used a carotid artery injury model. Denuding of the endothelial monolayer results in neointima formation and stenosis. Accelerated reendothelialization after injury diminishes neointima formation. Here, we show that exercise reduces neointima formation. We and others have recently demonstrated that vascular repair and reendothelialization after injury is enhanced by circulating EPCs.18,20,22 EPCs are incorporated into sites of active angiogenesis and have been shown to augment collateral vessel growth and to contribute significantly to adult blood vessel formation.13,29 Another important beneficial effect of physical exercise is the improvement of vascularization of ischemic tissue; for example, walking significantly improves collateral vessel formation in patients with peripheral vascular disease.7,8 Our experiments using the disk model of neoangiogenesis27 show a marked enhancement of new vessel formation in exercising mice. In this model, newly formed vessels were identified by transfusion of the mice with fluorescent microspheres into the left ventricle; therefore, only functional blood vessels connected to the circulation were detected. Given these data, we speculate that the increase in circulating EPCs induced by physical activity may contribute to the reduction of neointima formation and the enhancement of neoangiogenesis. However, additional and competing mechanisms participating in these obvious vascular benefits after training cannot be excluded.

In the present study, mice supplied with a running wheel ran voluntarily for several hours per day. It could be argued that our experimental model resembled natural conditions more closely than the usual sedentary lifestyle of laboratory mice. Mice subjected to forced exercise on a treadmill showed similar upregulation of EPCs. Therefore, it may be argued that the sedentary life of usual animal care represents an intervention that impairs vascular function. Indeed, a sedentary lifestyle in humans is a frequent modifiable risk factor associated with a 2-fold increased risk of coronary heart disease.1,2

To provide additional evidence for the biological significance of the findings, the studies were extended to humans. Our study shows that patients with coronary artery disease display increasing numbers of circulating EPCs in response to physical training, providing proof for the novel concept that EPC numbers can be increased by physical training. However, additional studies are needed to characterize whether and which forms of training can upregulate EPCs in healthy individuals.

Exercise improves symptoms and outcome of patients with coronary heart disease and heart failure. On the other hand, the potential vascular benefit of EPCs has been established. Interestingly, a recent study shows that EPC numbers correlate with endothelial function in humans.17 Thus, it may be speculated that the observed increase in EPCs may contribute to the molecular mechanisms underlying the well-documented advantages of physical activity on cardiovascular health.

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