Exercise Promotes Collateral Artery Growth Mediated by Monocytic Nitric Oxide


Objective—Collateral artery growth (arteriogenesis) is an important adaptive response to hampered arterial perfusion. It is unknown whether preventive physical exercise before limb ischemia can improve arteriogenesis and modulate mononuclear cell function. This study aimed at investigating the effects of endurance exercise before arterial occlusion on MNC function and collateral artery growth.

Approach and Results—After 3 weeks of voluntary treadmill exercise, ligation of the right femoral artery was performed in mice. Hindlimb perfusion immediately after surgery did not differ from sedentary mice. However, previous exercise improved perfusion restoration ≤7 days after femoral artery ligation, also when exercise was stopped at ligation. This was accompanied by an accumulation of peri-collateral macrophages and increased expression of endothelial nitric oxide synthase and inducible nitric oxide synthase (iNOS) in hindlimb collateral and in MNC of blood and spleen. Systemic monocyte and macrophage depletion by liposomal clodronate but not splenectomy attenuated exercise-induced perfusion restoration, collateral artery growth, peri-collateral macrophage accumulation, and upregulation of iNOS. iNOS-deficient mice did not show exercise-induced perfusion restoration. Transplantation of bone marrow–derived MNC from iNOS-deficient mice into wild-type animals inhibited exercise-induced collateral artery growth. In contrast to sedentary controls, thrice weekly aerobic exercise training for 6 months in humans increased peripheral blood MNC iNOS expression.

Conclusions—Circulating mononuclear cell–derived inducible nitric oxide is an important mediator of exercise-induced collateral artery growth. (Arterioscler Thromb Vasc Biol. 2015;35:1862-1871. DOI: 10.1161/ATVBAHA.115.305806.)

Key Words: bone marrow ■ collateral circulation ■ exercise ■ hindlimb ■ monocytes ■ nitric oxide ■ vascular growth

Physical exercise is a potent intervention for the primary and secondary prevention of vascular diseases. The extent of physical activity correlates with the protective effect.1 In stable peripheral arterial obstructive disease, exercise training is the only therapeutic measure receiving a class I recommendation in current guidelines.2 Several mechanisms have been proposed to underlie the beneficial effects of physical exercise for patients with ischemic disease, especially the improvement of endothelial cell function has been well documented.3 Collateral artery growth (arteriogenesis) is a natural escape mechanism to overcome the consequences of arterial obstruction or occlusion.4,5 The presence of coronary collateral arteries is associated with reduced cardiovascular mortality.6 Although there is some experimental evidence of increased collateralization after exercise,7 mechanisms involved are incompletely understood. Activation of endothelial nitric oxide synthase (eNOS)9 and increased release of mononuclear progenitor cells9 are among the molecular processes observed. Bone marrow (BM)–derived mononuclear cells (MNCs), particularly monocytes, orchestrate collateral artery growth.10 Exercise-induced release of NO in collateral vessels is thought to be largely shear stress–dependent. Whether there are exercise-induced effects beyond direct activation of the endothelium is currently unknown. For the clinical benefit after acute ischemia such as myocardial infarction11 or peripheral artery occlusion, arteriogenesis most likely must be initiated before the occlusion of the vessel. However, the effect of exercise on arteriogenesis in the absence of an arterial occlusion/obstruction has not been studied and potential underlying mechanisms are not known.

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In this study, we used a murine hindlimb model to investigate the effects of physical exercise before femoral artery occlusion. We then used monocyte depletion and BM transplantation studies to characterize the role of MNC for exercise-induced modulation of arteriogenesis. In a translational...
Nonstandard Abbreviations and Acronyms

eNOS  endothelial nitric oxide synthase
iNOS  inducible nitric oxide synthase
MNC  mononuclear cell
NO  nitric oxide

approach, inducible NO synthase (iNOS) expression of human MNC before and after 6 months aerobic exercise training was analyzed.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Exercise Before Femoral Artery Ligation Increased Arteriogenesis
Microsphere-assessed hindlimb perfusion fell to 3.5±0.7% of the unligated left control side acutely after right femoral artery ligation in sedentary mice. Mice offered a treadmill for voluntary physical exercise ran 4.0±0.3 km/d before and 0.5±0.2 km/d after unilateral femoral artery ligation. When ligating the right femoral artery after 3 weeks exercise, perfusion immediately after surgery was 4.0±0.8% of the unligated hindlimb (P=0.735 compared with sedentary). Thus, exercise did not influence baseline collateralization of the healthy murine hindlimb.

Seven days after ligation, hindlimb perfusion restoration as measured by fluorescent microsphere perfusion increased to 34.8±2.0% of the unligated hindlimb in control animals (sedentary). In mice exercising for 3 weeks before and until 1 week after ligation (run and run) perfusion restoration was increased to 52.8±4.1% (P<0.001 versus sedentary). In mice exercising for 3 weeks before ligation but without treadmill for the week after ligation (run and rest), perfusion restoration was similarly increased to 57.4±4.2% (P<0.001 versus sedentary; P=ns versus run and run). These hemodynamic findings were corroborated by immunohistochemical analyses showing an increase of the collateral arterial smooth muscle cell area as an indicator of cellular growth (smooth muscle cell constituting the most important proliferating cell type in arteriogenesis). Smooth muscle cell area increased from 26.2±3.3 in sedentary to 43.3±3.9 in run and run (P<0.001 versus sedentary) and 41.2±4.8 μm² in run and rest (P<0.001 versus sedentary; P=ns versus run and run). Similarly, the number of collateral arteries detectable in the main collateral-containing adductor muscle increased from 9.1±0.7 in sedentary to 16.7±0.6 per section in run and run (P<0.001 versus sedentary) and 17.3±0.9 in run and rest (P<0.001 versus sedentary; P=ns versus run and run). Because the running distance in the week post ligation was reduced to 0.5 km and no difference of the effect of run and run compared with run and rest on collateral blood flow were observed, we abstained from the group run and run in the following experiments. Time course analyses using Laser-Doppler imaging confirmed that exercise before femoral artery ligation increased perfusion restoration 3 and 7 days later. The prophylactic effect of exercise was upheld until 7 days after femoral artery ligation (and after exercise stop). Microfil-angiographies confirmed increased number and size of collateral arteries at day 7. In contrast, angiogenesis as assessed by calculating immunohistochemically analyzed capillary/fiber ratios remained unchanged (Figure 1).

To evaluate whether a positive effect of exercise was also detectable in a vascular disease model, the hypothesis was tested in an apolipoprotein E knockout (ApoE−/−) mice. Here, similar to wild-type mice, 3 weeks exercise training led to an increase in perfusion restoration 1 week after femoral artery ligation if assessed using fluorescent microsphere perfusion (exercise 54.15±5.4% versus sedentary 31.9±5.6% of the unligated left hindlimb; P=0.021).

Perivascular Macrophage Accumulation After Exercise
In flow cytometry, monocyte number after exercise did not increase compared with sedentary controls, but exercise seemed to inhibit increase in blood monocyte concentration after femoral artery ligation, possibly by increasing recruitment into perivascular tissue. Accordingly, the area of F4/80 positive peri-collateral macrophages in the adductor muscle increased after exercise to 195.0±17.6 versus 98.0±14.3 μm²; P<0.001, showing that exercise facilitates the accumulation of macrophages around collateral arteries (Figure 2).

NO Synthases Are Upregulated on Exercise and Femoral Artery Ligation
In large arteries, eNOS mRNA and protein expression are 3- to 4-fold upregulated after 3 weeks exercise. To test the hypothesis that local NO synthases may be important for arteriogenesis post exercise, the adductor muscles from ligated and nonligated limbs were compared. Protein expression and Ser1177 phosphorylation of eNOS (an indicator for activation) were not affected by exercise in pre-existing collateral arteries in the adductor muscle (eNOS/GAPDH 0.065±0.018 versus 0.072±0.023 [P=0.808], and phospho-eNOS/eNOS 3.040±0.373 versus 3.408±0.867 [P=0.702] in exercising versus sedentary mice). Similarly, Akt and phospho-Akt were not altered in collateral-containing hindlimb tissue. mRNA expression of inducible NOS was increased to 147±20% of sedentary (P=0.050). In contrast, when tissue was excised seven days after femoral artery ligation, phosphorylation of eNOS increased in exercised compared with sedentary controls (211±35%; P=0.034). iNOS mRNA expression was enhanced concomitantly to 229±47% of sedentary controls (P=0.011). Phosphorylated Akt was increased to 203±32% (P=0.019). [3H] citrulline–arginine converting assays showed enhanced NOS activity in collateral-containing adductor muscle compared with sedentary animals (131±11%; P=0.045). In immunohistochemistry, iNOS protein was colocalized to peri-collateral macrophages (Figure 3).

Source of iNOS and Role of Spleen
Next, systemic iNOS expression was investigated. In peripheral blood MNCs, iNOS mRNA was enhanced to 2387±572%
of sedentary controls ($P=0.025$). In the spleen, 7 days after femoral artery ligation, iNOS mRNA was enhanced to $226\pm60\%$ of sedentary controls ($P=0.035$). In BM MNCs of exercising animals, iNOS mRNA was increased to $417\pm139\%$ of sedentary controls ($P=0.062$). In summary, these data point toward a role of monocyte derived iNOS for the local environment of arteriogenesis.

We subsequently hypothesized that exercise-induced increase of peri-collateral macrophages and increased iNOS expression might originate from systemic—spleen-derived—MNCs. The experiments were, therefore, repeated with mice that underwent splenectomy before 3 weeks of running wheel exercise. In contrast to our hypothesis, microsphere perfusion 1 week after femoral artery ligation revealed that splenectomy did not alter arteriogenesis in sedentary mice (perfusion restoration $18.8\pm3.9$ versus $16.9\pm3.3$ [P=ns] of nonligated hindlimb in splenectomy versus control). In exercising mice postsplenectomy, perfusion restoration increased to $32.9\pm3.3\%$ of the nonligated hindlimb, compared with $31.3\pm3.3\%$ in non-splenectomized trained mice (P=ns), demonstrating that the exercise-induced increase was not affected by splenectomy (Figure 4).

**Monocyte and Macrophage Depletion Using Clodronate Liposomes Attenuates Exercise-Induced Stimulation of Arteriogenesis**

After the neutral effect of splenectomy on exercise-induced arteriogenesis, we hypothesized that systemic depletion of monocytes/macrophages may influence the positive effects of exercise on perfusion restoration. Because of the paucity of data on long-term effects of clodronate treatment, several series of experiments were performed comparing different injection intervals and concentrations. Peripheral blood monocyte count (flow cytometry) and macrophages in liver and spleen (immunohistochemistry) were used as readouts. An injection scheme using intravenous injections of $100 \mu L$ liposomes (0.5% clodronate) every 4 days proved to result in sustained depletion of both peripheral blood monocytes and macrophages in reticuloendothelial tissue (liver and
Weight gain as a parameter of general health remained unchanged compared with vehicle-injected control group during the treatment period. Running distance was comparable between placebo- and clodronate-treated animals. Perfusion restoration after femoral artery ligation as assessed using microsphere perfusion measurements was reduced in clodronate-treated (starting the day before ligation) animals to 61.3±6.2% of the nonligated hindlimb, as compared with 87.6±7.0% in control–liposome treated animals (*P*=0.008). In the above mentioned exercise model, perfusion restoration in the course of time was also assessed using laser Doppler fluxmetry. Placebo or clodronate liposomes were injected during the course of 3 weeks exercise and throughout 1 week after femoral artery ligation. The beneficial effect of exercise was annihilated in the group treated with clodronate liposomes, and perfusion restoration remained at the level of sedentary animals receiving vehicle (liposome) treatment. Corroborating the laser doppler imaging results, the increase in the collateral smooth muscle cell area was abrogated in clodronate-treated animals. Furthermore, comparable with the data in liver and spleen, clodronate liposomes strongly reduced the number of peri-collateral macrophages, resulting in a significant decrease of the macrophage area compared with vehicle-treated controls (Figure 5). In this model of arteriogenesis, capillary growth (CD31 immunostaining) in the adductor muscle was unaffected (data not shown).

**iNOS From Circulating Cells Is Needed for Monocyte Chemoattractant Protein-1-Expression and Exercise-Induced Prophylactic Effect on Arteriogenesis**

Mechanistically, iNOS mRNA expression was no longer increased by exercise in peripheral blood or spleen after macrophage depletion with clodronate. Similarly, in [3H] arginine–citrulline conversion assay, an increase in NO was detected in the collateral-containing adductor muscle after exercise that was no longer visible after monocyte depletion. Furthermore, monocyte depletion resulted in a loss of peri-collateral expression of the strong arteriogenic cytokine monocyte chemoattractant protein-1. In-vitro, after silencing of iNOS, monocyte chemoattractant protein-1-expression was reduced in MNCs (Figure 6). Thus, we hypothesized that the absence of monocytes and macrophages reduces the expression of iNOS that is an important contributor of exercise-induced prophylactic effect on perfusion restoration. To test this assumption, perfusion restoration after femoral artery ligation in exercising iNOS-deficient mice was measured.
sedentary iNOS-deficient animals, perfusion restoration as detected by microsphere perfusion experiments was mildly attenuated compared with wild-type animals post ligation. In contrast to wild-type mice, 3 weeks exercise did not increase perfusion restoration (28.4±2.8% of nonligated hindlimb in iNOS-deficient mice versus 57.4±4.2 in wild-type animals; P<0.001; Figure 7). Of note, in endothelial-NO synthase (eNOS) knockout mice, perfusion restoration was strongly attenuated in sedentary animals and could not sufficiently be stimulated by exercise either (8.0±0.8%; P<0.001 compared with wild-type).

As next step, BM from iNOS-deficient mice was transplanted into myoablated wild-type control mice. Animals ran similar distances as nontransplanted mice (data not shown). In exercising mice with iNOS-deficient BM perfusion restoration after exercise and femoral artery ligation was attenuated compared with controls (wild-type mice receiving BM from wild-type animals; 34.3±5.1 versus 19.8±2.1% perfusion of ligated versus nonligated hindlimb; P=0.013), demonstrating circulating MNC to be the source for iNOS (Figure 7).

**Aerobic Exercise Training in Humans Increases Circulating MNC iNOS Expression**

Translating the findings into the human situation, healthy volunteers (mean age, 49±7; 40% men) underwent controlled aerobic exercise training (45 minutes, 3× a week) for 6 months. Volunteers had initially been randomized into this training or a control group. iNOS mRNA expression of Ficoll-isolated peripheral blood MNC was increased to 279±65% of pretraining levels (P=0.039; Figure 8). Controls not undergoing supervised training demonstrated no change after 6 months (101±21%; P=0.982 compared with baseline). Plasma levels of MCP1 (monocyte chemoattractant protein-1) remained unchanged (before/after 6 months training: 368±51 pg/mL/358±40 pg/mL, compared with controls 377±54 pg/mL/351±49 pg/mL).

**Discussion**

This is the first investigation reporting on the detailed cellular mechanisms of exercise-induced collateral artery growth. The main novel finding is that inducible NO synthase from BM-derived circulating monocytes is critical for the beneficial...
Here, these data are extended demonstrating a specific role of physical exercise.3,9,15,16 Exercise-mediated enhanced shear stress is an important mediator of increased expression and activity of endothelial NO synthase in conductance arteries.17 Effects of exercise on perfusion restoration after femoral artery ligation.

NO Is a Mediator of Exercise-Induced Arteriogenesis

Arteriogenesis represents a key mechanism of compensation for the reduced tissue flow caused by an obstructed artery.11 However, effective therapeutic strategies to improve collateral artery formation remain limited demonstrating the need for further mechanistic insight. This study finds that voluntary physical activity compared with the typical sedentary lifestyle of laboratory animals provides powerful improvement of the restoration of blood flow after hindlimb ischemia. An earlier investigation in rats has reported that treadmill running before femoral artery ligation acutely increased blood flow,14 an effect that is most likely mediated by vasomotor tone. In this study, careful assessment of perfusion under conditions of maximal vasodilation showed that previous exercise training does not increase the blood flow acutely after femoral artery ligation but promotes arteriogenesis that subsequently results in an accelerated restoration of blood flow in the following days.

Further study of the underlying molecular mechanisms revealed an important role of NO in the ischemic tissue. In large arteries, increased endothelial NO bioavailability is a hallmark of physical exercise.9,18 Exercise-mediated enhanced shear stress is an important mediator of increased expression and activity of endothelial NO synthase in conductance arteries.17 Here, these data are extended demonstrating a specific role of the inducible NOS for arteriogenesis in a different cellular compartment, the peri-collateral tissue. The source of iNOS is circulating MNCs because both clodronate-induced depletion of monocytes/macrophages and MNC–specific depletion of iNOS abrogates exercise-induced collateral artery growth. In contrast to the shear stress–induced upregulation of eNOS in the murine aorta, eNOS activity remained unchanged in the collateral-bearing hindlimb adductor muscle probably because no increase in shear stress is to be expected in pre-existing collateral anastomoses as long as the femoral artery is fully patent. In contrast, iNOS upregulation was detected after exercise in collateral tissue independent of femoral artery ligation, pointing to a shear stress–independent mechanism. After ligation, iNOS expression remained consistently upregulated in the MNCs surrounding the collateral arteries. Besides a direct vascular effect of NO from iNOS, we provide evidence of an attenuation of arteriogenic monocyte chemoattractant protein-1 after iNOS depletion, thus possibly leading to a further inhibited monocyte recruitment. In a translational approach, iNOS upregulation was also found in MNCs of healthy humans after 6 months endurance exercise training. Recent work also points to a monocyte-modulating effect of regular exercise in intermittent claudication.18

Shear stress is a major driving force for collateral artery growth. Physical exercise increases shear stress in the pre-existing collateral circulation after obstruction/occlusion of a large artery.19 At the same time, the critical role of circulating monocytes in arteriogenesis is widely appreciated.10 The findings of this study provide a link between the concepts of exercise-induced increase in collateral artery growth and the role of MNCs in arteriogenesis. The observed prophylactic effect seems not only shear stress–dependent. Rather, exercise upregulated iNOS in BM–derived circulating cells that invade into peri-collateral tissue to act as the source of NO.

It should be noted that the effect of NO on vascular growth20 and myocardial blood flow21 has been discussed controversially in the literature. Uncoupling of the eNOS enzyme can also aggravate oxidative stress.22 Also, left ventricular remodeling after myocardial infarction is described to be dependent on NO.23

Macrophages Are a Source of Enhanced NO Levels

Macrophages accumulate around growing collateral arteriies24 and are critically involved in arteriogenesis. Their causal role for the growth of pre-existent collateral anastomoses has been studied using mice deficient of the macrophage colony-stimulating factor25 or after monocyte depletion using 5-fluorouracil.26 These interventions not only reduce the number of macrophages but also affect growth factors and systemic inflammation at the same time. For example, application of 5-fluorouracil results in a marked rebound effect in addition to a distinct antiproliferative effect of the compound. The monocyte/macrophage depletion technique by liposomal clodronate used in this investigation has the advantage to induce a long-term depletion of monocytes and macrophages. As a potential limitation the inflammatory reaction caused by regular liposome injections might influence the vascular growth process, as reflected by increased perfusion restoration in
the liposome-vehicle group. We elaborated different dosing schemes establishing a protocol that yielded sufficient depletion of monocytes in the blood and in tissue macrophages, which resulted in a highly reproducible decrease of perfusion recovery both assessed using microsphere perfusion and laser Doppler measurements. The source of MNCs contributing to enhanced arteriogenesis has hitherto been unknown. Spleen-derived MNCs play a major role in vascular remodeling.

Figure 5. Monocyte depletion by clodronate liposomes reduces perfusion restoration. Intravenous injections of 100 μL clodronate liposomes as shown in (A) demonstrate reduction of circulating monocytes as assessed in flow cytometry compared with placebo (clodronate-free liposomes) injections, which result in reactive increase of circulating monocytes (n=10 each). The effect is upheld ≤72 hours post injection. Representative scatter plots are shown in (B). Macrophages in liver and spleen, stained by F4/80, were diminished 48 hours after clodronate injection (C). Assessing perfusion restoration using fluorescent microsphere perfusion 1 week after femoral artery ligation in mice resulted in decreased hindlimb perfusion in animals (n=10 per group), which received clodronate injections the day before and 3 days after femoral artery ligation (D). Next, both sedentary and exercising mice underwent clodronate injections once in 4 days as indicated on the time bar (E). Three weeks exercise before femoral artery ligation increased perfusion restoration 7 days after femoral artery ligation as already shown in Figure 1. When clodronate liposomes were injected during exercise, the positive influence of exercise before ligation was abolished (F, n=10 per group). Representative laser Doppler images showing this lack of exercise-induced perfusion increase in monocyte-depleted mice 7 days after femoral artery ligation are shown in (G). Exercise-induced increase of collateral smooth muscle cell area as a measure of vessel size was attenuated by clodronate (H). In resting animals, clodronate reduced the number of peri-collateral macrophages. Also, exercise-induced increase of macrophages was no longer visible when clodronate was injected together with exercise (I). Representative immunohistochemical pictures of macrophages (red) around collateral arteries (smooth muscle actin staining, green) are shown in (J). Inducible NO synthase (blue) detected in macrophages (red; purple color indicates co-staining of red macrophages and blue iNOS) around collateral arteries (green) was reduced after clodronate treatment, as shown in confocal microscopy (K). SMC indicates smooth muscle cell.
post myocardial infarction.28 There, splenic accumulation of inflammatory monocytes in myocardial infarction and release toward the myocardium was associated with myocardial remodeling. Different subtypes of monocytes display both detrimental properties leading to maladaptive remodeling, and beneficial features yielding to adaptive responses, such as angiogenesis.29 We hypothesized that depletion of spleen-derived cells would eliminate a potential source of these cells, thereby impeding their effect on arteriogenesis. However, the data obtained post splenectomy show that spleen-derived cells are not of major relevance for adaptive vascular remodeling. Subsequent BM transplantation studies revealed that

Figure 6. Inducible nitric oxide synthase (iNOS) is attenuated by clodronate and mediates monocyte chemoattractant protein-1 (MCP1) expression. Exercise-induced increase in mRNA expression of iNOS was no longer present in peripheral blood mononuclear cells (PBMNC, A) or spleen-derived mononuclear cells (B) when the animals (n=10) were treated with clodronate during the exercise period. Measuring the amount of bioavailable nitric oxide in arginine-citrulline conversion assays showed that exercise-induced arginine availability decreased after clodronate (C). In these mice, gene expression of the arteriogenic cytokine MCP1 was strongly reduced. Mononuclear THP1 cells were treated with siRNA against iNOS in vitro (n=6 per group). Successful transfection is indicated by green fluorescent protein-coupled siRNA in (E). After transfection, MCP1 mRNA expression was significantly reduced, showing a link between iNOS and this important proarteriogenic cytokine (F).

post myocardial infarction.28 There, splenic accumulation of inflammatory monocytes in myocardial infarction and release toward the myocardium was associated with myocardial remodeling. Different subtypes of monocytes display both detrimental properties leading to maladaptive remodeling, and beneficial features yielding to adaptive responses, such as angiogenesis.29 We hypothesized that depletion of spleen-derived cells would eliminate a potential source of these cells, thereby impeding their effect on arteriogenesis. However, the data obtained post splenectomy show that spleen-derived cells are not of major relevance for adaptive vascular remodeling. Subsequent BM transplantation studies revealed that

Figure 7. Inducible NO synthase from circulating cells mediates exercise induced microsphere assessed perfusion restoration. Compared with wild-type (WT) mice (A), mice lacking inducible nitric oxide (iNOS, B) failed to show an exercise-induced increase in arteriogenesis (n=7 per group). In comparison, mice lacking endothelial NO synthase (eNOS, C), already had strongly attenuated perfusion restoration at rest, which was mildly increased by exercise (n=8 each). Bone marrow transplantation experiments from iNOS−/− to WT mice (D) showed that irradiated WT mice, which received bone marrow from iNOS−/− animals exhibited much weaker exercise-induced perfusion restoration after femoral artery ligation (E, n=10 each).
mice with iNOS-deficient BM do not show increased collateral artery growth after exercise. These experiments identify BM-derived MNCs to be the source of iNOS necessary for the exercise-induced increase in arteriogenesis. In line with previous reports, blood monocyte concentration dramatically dropped after femoral artery ligation, suggesting increased tissue recruitment of these cells. Both source, location, and particularly time course of activation of inflammatory cells must be critically controlled during vascular growth. Excess inflammation, similarly to excess activation of NO signaling, is known to inhibit rather than stimulate vascular growth.31,32 These data have limitations as they were, for the largest part, obtained from healthy experimental animals. The pro-arteriogenic effect of voluntary exercise was confirmed in the vascular disease model of ApoE knockout mice. The so far premature clinical data, however, need to be further scrutinized in monocytes from patients with peripheral arterial disease. Furthermore, investigating humoral factors in a human study is always subject to interindividual variation and influences of other variables not covered by the study protocol.

As an outlook, these findings may not be limited to collateralization of the peripheral circulation. Other groups have alluded to a beneficial effect of exercise on coronary33 or cerebrovascular34 vascular growth. In these vascular territories, exact mechanisms of exercise-induced vascular growth are yet to be elucidated and may be comparable with those identified here. A relevant role of perivascular inNOS in arteriogenesis has earlier been demonstrated.35 Of note, during remodeling after myocardial infarction, classical inflammatory macrophages (as a source of iNOS) are the predominant cell type only in the early phase (day 2), whereas later (day 5), alternatively activated macrophages prevail.36,37 Macrophage subtypes during hindlimb arteriogenesis also seem to follow a temporal and spatial distribution, where both subtypes seem necessary during different phases of collateral artery growth.38 The identification of circulating monocytes as the source for a proarteriogenic factor (iNOS) might set the stage for novel ideas for future therapeutic approaches. Because monocytes can be modified ex-vivo and reinfused, use of these cells as carriers to deliver a proarteriogenic strategy into the vasculature could be feasible.39 Monocyte-mediated iNOS at the right time (after arterial occlusion) and in the right cellular compartment (ischemic perivascular tissues) could be a potential future target. In the meantime, our data strongly support exercise prescription as an essential strategy of prevention and treatment for patients with peripheral arterial disease.

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Disclosures

None.

References


Significance

Physical training is a cornerstone in vascular prevention. Its exact modes of action are unclear. Understanding the underlying mechanisms provides further support for this approach. Here, we show that endurance exercise in a murine model not only is beneficially after vascular occlusion, when increased shear stress activates the collateral endothelium, but also stimulates vascular growth in a prophylactic manner, that is, before vascular occlusion. Nitric oxide from bone marrow–derived monocytes mediates this process, as demonstrated by monocyte/macroage depletion and bone marrow transplantation experiments from inducible nitric oxide synthase knockout mice. Also in humans, inducible nitric oxide synthase was increased after endurance exercise. These data provide new insights into mechanisms of collateral artery growth. Serving as mediator, circulating monocytes might be used in novel experimental approaches to stimulate vascular growth, possibly after modification to express higher amounts of nitric oxide.
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Supplemental Material

Methods

Animals, exercise

Animal experiments were approved by the local authorities. Ten weeks old male mice were divided into a sedentary control group and exercising animals. Exercising mice were supplied with running wheels. Mice ran voluntarily as described (1,2).

Femoral artery ligation

A total of 206 C57Bl/6N and 14 iNOS−/− mice were subjected to femoral artery ligation (FAL) as described (3). Briefly, after a 5 mm incision below the inguinal ligament, the right femoral artery was dissected and ligated twice just distally to the branch of the deep femoral artery, thereby insuring sufficient collateral blood flow at rest to prevent tissue necrosis in the lower leg.

Splenectomy

C57Bl/6N mice were subjected to splenectomy. Using a small left-lateral incision below the thorax, splenic artery and vein were dissected, carefully ligated, and the spleen was excised.

Microsphere perfusion and Microfil® angiography

Mice underwent collateral-dependent perfusion measurements of the lower extremities seven days after FAL using fluorescent microspheres(4). Post-mortem angiograms of mouse hindlimbs were produced using Microfil® followed by alcohol dehydration and formaldehyde fixation as previously described (3).

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imager was used to assess the limb perfusion as described (5). Directly before, after, and 3-28 days after FAL, laser Doppler intensities of the foot area, where adequate penetration depth of the laser is ensured, were recorded and expressed as ratio ligated to non-ligated hindlimb.

Flow cytometry

Flow cytometry was used for the assessment of peripheral blood monocytes, which were identified as CD11b and CD115 and gated accordingly in flow cytometry.
Immunohistochemistry

Histological sections (5 µm) were prepared from hindlimb tissue (adductor muscle). Perivascular macrophages (anti-F4/80), vascular smooth muscle cells (anti-smooth muscle actin), capillaries (anti-CD31), and iNOS (anti iNOS) were stained immunohistochemically for confocal microscopy. To better display all structures, confocal microscopic images were taken in different layers. Since confocal images have a thickness of less than 1 µm, macrophages and blood vessels had to be merged from planes within the same thin section. Automated, computer-assisted quantification of area was performed to minimize variability.

Gene and protein expression analysis

Ficoll® density gradient centrifugation was used to obtain mononuclear cells from peripheral whole blood, bone marrow and lysed spleen tissue. mRNA expression of mm18S rRNA and iNOS was assessed of mononuclear cells from peripheral blood, bone marrow and splenic tissue as well of hindlimb tissue. MCP1 (monocyte chemoattractant protein-1) mRNA expression was measured in hindlimb tissue. Immunoblotting for eNOS and phospho-eNOS (Ser1177) was performed from hindlimb tissue.

Quantification of tissue nitric oxide

In hindlimb tissue, NOS activity was measured using a radioactive [³H]-arginine-citrulline assay (3).

Monocyte/macrophage depletion

Clodronate liposomes have been used extensively to specifically deplete macrophages in vivo by inducing apoptosis in these cells without affecting other cell types. Clodronate was supplied by Roche and clodronate liposomes were prepared as described previously (6).

Bone marrow transplantation

Eight weeks old C57Bl/6 wildtype mice, and iNOS knockout mice (iNOS⁻/⁻ B6.129P2-Nos2₉₃₅Lau/J) were sacrificed and bone marrow was obtained from the long bones. BM cells (1–2 x 10⁷) were transplanted into 10-week-old male, lethally irradiated (total dose 9 Gy), recipient wildtype mice by injection into the retroocular vein plexus 5 h after irradiation. Four weeks after BM transplantation (BMT), mice to undergo exercise were again allowed to physically exercise on a treadmill for three weeks, after which FAL was performed.

MNC iNOS and plasma CCL2 expression in human volunteers

Human volunteers underwent a supervised aerobic exercise program consisting of three 45 minute exercise units thrice a week. Blood was drawn for Ficoll® density gradient centrifugation before and after the 6 months period (two days after last training). Volunteers not undergoing
training served as controls. Mononuclear cells were lysed, and real-time PCR was used to quantify iNOS mRNA expression in MNC. CCL2 (monocyte chemoattractant protein-1, MCP1) was measured using commercially available ELISA (R&D systems) from all volunteers before and after exercise.

**Cell culture and RNA silencing experiments**

Mononuclear THP1 cells were cultured in standard medium (RPMI) and transfected with control or iNOS siRNA, which was green-fluorescent protein (GFP) labelled to visualize successful transfection. Following lysis and mRNA isolation, MCP1 mRNA expression was quantified using real-time PCR.

**Statistics**

Results are presented as mean±standard error of the mean. Student’s t-test was used to compare two groups with normally distributed parameters. A p-value of <0.05 was considered statistically significant. When comparing three or more groups, analysis of variance with post-hoc Bonferroni correction was performed. SPSS 18.0 was used to compute statistical significances.

**References:**