Physical Activity Improves Long-Term Stroke Outcome via Endothelial Nitric Oxide Synthase–Dependent Augmentation of Neovascularization and Cerebral Blood Flow

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Abstract—Physical activity upregulates endothelial nitric oxide synthase (eNOS), improves endothelium function, and protects from vascular disease. Here, we tested whether voluntary running would enhance neovascularization and long-term recovery following mild brain ischemia. Wild-type mice were exposed to 30 minutes of middle-cerebral artery occlusion (MCAo) and reperfusion. Continuous voluntary running on wheels conferred long-term upregulation of eNOS in the vasculature and of endothelial progenitor cells (EPCs) in the spleen and bone marrow (BM). This was associated with higher numbers of circulating EPCs in the blood and enhanced neovascularization. Moreover, engraftment of Tie2/LacZ-positive BM-derived cells was increased in the ischemic brain. Four weeks after the insult, trained animals showed higher numbers of newly generated cells in vascular sites, increased density of perfused microvessels and sustained augmentation of cerebral blood flow within the ischemic striatum. Moreover, running conferred tissue sparing and improved functional outcome at 4 weeks. The protective effects of running on angiogenesis and outcome were completely abolished when animals were treated with a NOS inhibitor or the antiangiogenic compound endostatin after brain ischemia, and in animals lacking eNOS expression. Voluntary physical activity improves long-term stroke outcome by eNOS-dependent mechanisms related to improved angiogenesis and cerebral blood flow. (Circ Res. 2006;99:1132-1140.)

Key Words: angiogenesis ■ cerebral ischemia ■ exercise ■ nitric oxide synthase

Regular physical activity is associated with decreased incidence of cerebro- and cardiovascular events.1,2 Although the molecular mechanism(s) of these protective effects are incompletely understood, it has become apparent that physical training improves endothelium-dependent vasodilation, in part via upregulation and increased phosphorylation of endothelial nitric oxide synthase (eNOS).3,4 We have recently demonstrated that running acutely protects brain tissue after ischemia by eNOS-dependent mechanisms.5 However, it is presently unclear whether physical activity also improves long-term recovery and regeneration after brain ischemia.

Clinical and experimental evidence suggests that brain ischemia promotes the formation of new vessels.6 In general, neovascularization can take the form of angiogenesis (ie, the generation of new vessels from existing vasculature), arteriogenesis, or postnatal vasculogenesis mediated by mobilization of stem and progenitor cells.7 A specific subset of bone marrow (BM)-derived cells, endothelial progenitor cells (EPCs), may promote vascular repair, neovascularization, and improve endothelial function.8,9 The functional impact of EPCs on vessel formation, cerebral blood flow (CBF), and tissue recovery in the ischemic brain, however, is not clear.10–13

We have recently demonstrated that physical training enhances neovascularization and increases EPCs by an eNOS-dependent mechanism.9 Interestingly, eNOS also modulates new vessel formation by tissue ischemia, is critical for ischemic remodeling and for mobilization of stem and progenitor cells, and even upregulates neurogenesis in the brain.5,9,10,14 Here, we tested the hypothesis that regular physical activity increases neovascularization and augments chronic CBF, thereby improving long-term recovery in a well-characterized mouse model of mild stroke.

Materials and Methods

Animals and Treatment

All procedures were approved by an official committee. Male 129S6/SvEv mice (BR, Berlin, Germany), C57Bl/6J mice (ie, background strain for eNOS-deficient mice; The Jackson Labora-
Cerebral Ischemia and Measurement of Physiological Parameters

Mice were anesthetized with 1.0 volume percent isoflurane in 69% N2O and 30% O₂ and subjected to left MCAo for 30 minutes followed by reperfusion as described. Regional CBF measurements were performed using the 14C-iodoantipyrine technique under etomidate anesthesia (0.03 mg/kg body weight per minute; Braun, Melsungen, Germany) as described. Density of Perfused Vessels

Evans blue (2% in saline; Sigma-Aldrich) was administered intravenously and allowed to circulate for 5 minutes under etomidate anesthesia. In some animals acetazolamide (ACZ) (Diamox, 30 mg/kg body weight; Sigma-Aldrich) was coadministered intravenously 5 minutes before euthanasia. Animals were decapitated and brains cut into 10-μm coronal cryostat sections and digitized with a cooled charge-coupled device camera (Dage-MTI, Michigan City, Ind) with a fluorescence microscope equipped with a ×10 objective. Single camera images were joined together with the technique of tiled-field mapping (MCID Elite, InterFocus, Mering, Germany). Regions of interest were specified with the technique of density slicing, including the setting of target acceptance criteria.

Histochimistry and Immunohistochemistry

Brains were perfusion fixed with 4% paraformaldehyde and cut into 40-μm sections. For X-gal histochemistry 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma-Aldrich) was dissolved in dimethylformamide (Sigma-Aldrich) and further diluted in staining solution consisting of 50 mmol/L potassium ferricyanide, 50 mmol/L potassium ferrocyanide, and 1 mol/L magnesium chloride in PBS to a final concentration of 1 mg X-Gal/1 mL staining solution. BrdUrd immunohistochemistry was performed as described previously. Primary antibodies were anti-BrdUrd (rat, 1:500; Biozol, Eching, Germany) and anti–von Willebrand factor (anti-vWF) (rabbit, 1:200; Chemicon, Temecula, Calif). Secondary antibodies used were anti-rat Rhodamine X, anti-rabbit FITC, and anti-rat and anti-rabbit Biotin SP (all 1:250; Dianova, Hamburg, Germany). Confocal microscopy was performed using a spectral confocal microscope (TCS SP2; Leica).

Lesion Determination

Cerebral lesion volumes were determined on 20-μm cryostat sections as previously described.

Functional Outcome

Sensory-motor deficit scores were determined as described. Endurance in the “wire-hanging test” was measured on a horizontal steel wire (1 mm) stretched horizontally 50 cm above a foam pad. A full experiment of Morris water maze consisted of a “place task” with three trials per day for 7 consecutive days and a “probe trial” (“spatial probe”) on day 8 as described.

Statistical Analysis

Data are presented as mean±SEM. Comparisons were made by 1- or 2-way ANOVA or repeated-measures ANOVA followed by Tukey's post hoc test.
Results

Running Confers Long-Term Histological and Functional Protection Following Mild Stroke

Cerebral lesion sizes at 4 weeks were reduced by 38% in running versus sedentary 129/SV mice (Figure 1A). Similarly, lesion volumes were also reduced by 44% in C57Bl/6J mice (ie, background strain for eNOS-deficient mice; 16.0±1.8 versus 8.9±1.7 mm³; P<0.05). Subgroups of sedentary and running 129/SV mice were treated with L-NAME or with endostatin for 14 days starting 1 day after MCAo. Both L-NAME and endostatin completely abolished the protective effect of physical activity at 4 weeks (Figure 1A). There was also no protective effect of running on lesion volume in eNOS-deficient mice (20.1±3.7 versus 21.4±3.2 mm³). In 129/SV mice exposed to running wheels only after brain ischemia (poststroke running), cerebral lesion volumes were reduced by 18% at 4 weeks; however, this result did not reach statistical significance (P=0.08; Figure 1B).

Laser Doppler measurements during MCAo indicated that the reductions in regional CBF in ischemic core areas were similar in all groups (see Materials and Methods). Moreover, physiological parameters did not differ significantly between anesthetized running versus sedentary animals, neither during MCAo nor at 4 weeks (mean arterial blood pressure [MABP], 123±12 versus 109±5 mm Hg; pH, 7.31±0.02 versus 7.27±0.01; PCO₂, 50±1 versus 49±1 mm Hg; PO₂, 87±9 versus 88±2 mm Hg in sedentary versus running mice; P>0.05; n=6 per group). In addition, we performed telemetric recordings in awake mice over a 3-day period at 4 weeks after MCAo (n=4 per group). Average blood pressure (BP) and HR were not different in running (diastolic BP, 95±15 mm Hg; MABP, 103±15 mm Hg; systolic BP, 116±32 mm Hg; HR, 488±51 bpm) as compared with sedentary (diastolic BP, 96±8 mm Hg; MABP, 107±9 mm Hg; systolic BP, 121±32 mm Hg; HR, 491±50 bpm) mice. Also, the circadian rhythms were not significantly altered by running (Figure 1C).

In addition, running also conferred long-term functional and cognitive improvement: sensory-motor deficit scores and endurance in the wire-hanging test were significantly improved in running compared with sedentary 129/SV mice (Figure 1D and 1E). For assessment of spatial learning, animals were tested in the Morris water maze. In the place task (acquisition; day 1 to 7), latencies to find the platform tended to be better in the running versus sedentary group (Figure 1F). In the probe trial (retention; day 8), running animals visited the target zone significantly more often than sedentary mice, whereas speed and total distance did not differ between groups (Figure 1G). In addition, runners but not sedentary animals visited the former target zone more frequently in the first versus second minute, indicative of successful strategy-switching.20

Effects of Running on eNOS, Neovascularization, and EPCs Following Cerebral Ischemia

Previously, we demonstrated that voluntary running for 3 weeks upregulated aortic eNOS mRNA, protein, and activity by ~3- to 4-fold.5 Following MCAo, eNOS mRNA expression in aortas remained significantly upregulated in runners at...
10 days (by 2.3-fold) and 4 weeks (by 3.1-fold), although MCAo itself did not have an effect on eNOS mRNA expression (Figure 2A). Moreover, running also increased eNOS expression in EPCs by 1.6-fold, as measured by Western blotting at 3 weeks (Figure 2B). VEGF serum levels were elevated in runners and sedentary animals following MCAo compared with sham at day 10, whereas by 4 weeks, VEGF levels tended to be higher in only the running group (Figure 2C). In addition, the systemic angiogenic response was determined in a neovascularization bioassay that depends on eNOS activity. Running significantly increased the area of neovascularization compared with sedentary mice, with no differences between sham and MCAo animals (Figure 2D and 2E).

Because eNOS plays a role in EPC regulation, EPCs were quantified in BM and peripheral blood by fluorescence-activated cell sorting (FACS) analysis. Running increased EPCs in BM at days 2 and 10 following MCAo compared with sedentary mice, without differences between sham and MCAo (Figure 3A). In peripheral blood, both running and ischemia increased EPCs at days 2 and 10 (Figure 3B). In addition, mononuclear cells were isolated from spleen and cultured under EPC-specific conditions. EPC numbers were assessed after staining with Dil-Ac-LDL and lectin. Mean ± SEM; n=5 per group; *P<0.05 vs sham, #P<0.05 vs sedentary; 2-way ANOVA and Tukey post hoc test.

**Figure 2.** Effects of running and mild ischemia on eNOS expression in aortas and EPCs, VEGF serum levels, and disc neovascularization. A, eNOS expression was determined by RT-PCR in aortas of running and sedentary mice 10 days and 4 weeks after 30 minutes of MCAo/reperfusion or after sham operation. Mean ± SEM; n=5 per group; *P<0.05 vs sedentary, ANOVA and Tukey’s post hoc test. B, Immunoblots (20 µg of protein per lane) showing eNOS protein expression in spleen-derived EPCs from sedentary and running mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal standard. Mean ± SEM; n=5 per group; *P<0.05 vs sedentary, Student’s t test. C, VEGF serum levels were measured by ELISA in running and sedentary mice 2 days and 4 weeks after 30 minutes of MCAo/reperfusion or sham operation. Mean ± SEM; n=5 per group. D, Following 30 minutes of MCAo/reperfusion or sham operation, polyvinyl sponges were implanted subcutaneously. Running was continued and in-growth of new vessels quantified after 2 weeks. Mean ± SEM; n=10 animals per group; *P<0.05 vs sedentary, ANOVA and Tukey’s post hoc test. E, Representative examples of vascularized area around border zone of discs.

**Figure 3.** Sca-1/VEGFR2+ cells were quantified by FACS analyses in samples of BM (A) and peripheral blood (B) of running and sedentary mice at 2 and 10 days after 30 minutes of MCAo/reperfusion or sham (S) operation. Mean ± SEM; n=5 per group; *P<0.05 vs sham, #P<0.05 vs sedentary; 2-way ANOVA and Tukey post hoc test.
EPCs only at day 2 (Figure 3C). Angiogenic response and increases in EPC levels were blunted when animals were cotreated with L-NAME.9

Using BM chimeric mice that express β-galactosidase under control of the TIE2 promoter in BM cells, the recruitment of TIE2-expressing (TIE2/LacZ) BM-derived cells into the ischemic brain was determined.11,12 In sham-operated animals, we observed only few TIE2/LacZ cells, which were associated with the vasculature (not shown). Four days following 30 minutes of MCAo/reperfusion, TIE2/LacZ cell density was significantly higher in the ischemic versus contralateral striatum, and this was augmented in running compared with sedentary mice (Figure 4A and 4C). Increased TIE2/LacZ cell numbers were sustained for at least 3 weeks after MCAo (not shown). Some animals received an intravenous injection with FITC-lectin 5 minutes before euthanasia. Within the ischemic striatum, TIE2/LacZ cells were exclusively found lining lectin-stained vessels, suggesting that BM-derived cells participate in the angiogenic response (Figure 4B).

Running Increases Angiogenesis, Density of Perfused Microvessels, and CBF

Proliferating cells were pulse-labeled with the S-phase marker BrdUrd. When animals received 5 daily consecutive BrdUrd injections before MCAo, there were only low numbers of BrdUrd+ cells within ischemic tissue at 4 weeks, which were elevated in runners (944±16, 976±17, and 1329±75 BrdUrd+ cells/mm3 in sham, sedentary/ MCAo, and runners/MCAo, respectively). When animals received 5 daily BrdUrd injections starting 1 day after MCAo, BrdUrd+ cell density 4 weeks later was significantly higher in the ischemic striatum compared with sham and further increased in running compared with sedentary animals (Figure 5A). Similarly, the number of vessel (vWF)-associated BrdUrd+ cells was significantly higher in running versus sedentary animals (≈1300/mm3 versus ≈70/mm3; Figure 5B). When animals were cotreated with the angiogenesis inhibitor endostatin for 14 days starting 1 day after MCAo, the effect of running on BrdUrd+ and
vWF-associated BrdUrd− cells was completely abrogated (Figure 5A and 5B). vWF immunostaining demonstrated largely increased endothelial cell densities within ischemic tissue of running compared with sedentary mice, and this again was completely abrogated in animals cotreated with endostatin or with L-NAME (Figure 5C and 5D).

The density and caliber of perfused microvessels was quantified at 4 weeks using endovascular Evans blue staining and tiled-field imaging (Figure 6A through 6D). MABP during Evans blue administration was 107±3 mm Hg (sedentary), 110±4 mm Hg (running), and 108±3 mm Hg (running/endostatin). Sedentary animals showed grossly enlarged microvessels within ischemic tissue compared with sham or contralateral side (Figure 6B through 6D). In runners, average vessel caliber was not different from sham or contralateral side, and the density of perfused vessels was significantly higher compared with the sedentary group (Figure 6A and 6C). In animals treated with endostatin for 14 days after MCAo, the effects of running on both vessel density and caliber were completely abolished.

In additional mice, ACZ was administered before euthanasia to induce vasodilation. In the contralateral striatum average vessel caliber was increased in both sedentary (∼65% increase) and running mice (∼60% increase). In contrast, whereas average caliber was increased by 30% in the ischemic striatum of running mice, it decreased by 17% in the ischemic striatum of sedentary mice.

Moreover, we categorized vessels according to their diameter (ranges: <12, 12 to 20, 20 to 50, and >50 μm). Figure 6E presents the percentage of all vessels within each diameter category. In sedentary mice, the density of vessels within category <12 μm were decreased, whereas those >20 μm were increased in the ischemic striatum compared with contralateral side. In contrast, running mice had significantly
higher density of vessels <12 μm but lower density of those 20 to 50 μm in the ischemic striatum compared with sedentary mice. ACZ significantly affected vessel categories in the contralateral striatum of both sedentary and running mice. In contrast, in ischemic striatum of sedentary mice ACZ had no effect on vessel categories, whereas there was a significant effect in running mice (Figure 6E).

Absolute regional CBF (rCBF) was significantly lower in the ischemic striatum compared with contralateral side in sedentary mice at 4 weeks (Figure 7). In runners, rCBF was significantly higher both in the ischemic and contralateral striatum compared with sedentary animals, an effect that was again completely abolished after endostatin cotreatment (Figure 7). In sham-operated mice, we detected no significant CBF alterations (data not shown).

Discussion
This study has the following major findings. In a well-characterized mouse model of mild brain ischemia, voluntary running upregulates eNOS expression and increases the number of EPCs in BM and spleen, which is further enhanced by brain ischemia. This correlates to higher numbers of EPCs circulating in the blood and enhanced neovascularization in a disc angiogenesis model. Moreover, engraftment of TIE2/LacZ⁺ BM cells was increased in the ischemic brain. At 4 weeks, running significantly enhances the number of newly generated cells at vascular sites, increases the density of perfused microvessels with apparently normal morphology, and augments absolute rCBF in the ischemic lesion. The proangiogenic effects of running are associated with improved outcome, as evidenced by lesion sparing and better functional and cognitive outcome. There are no apparent changes in physiological parameter that could explain these effects. Rather, upregulation of eNOS and the resulting angiogenesis appear to mediate the protective effects of regular physical activity because the effects on angiogenesis, vessel density, and blood flow, as well as on outcome, were completely abolished when animals were either cotreated with a NOS inhibitor or an angiogenesis inhibitor, endostatin, as well as in animals lacking eNOS gene expression (Figure 8).

We focused on the mechanisms of pre- plus poststroke voluntary running on recovery. When animals were trained only after ischemia, there was a nonsignificant trend toward smaller lesions, which was consistent with the literature. So far, most stroke studies have focused on acute outcome, and there are treatment paradigms in which beneficial effects are lost over time. In this study, we have linked the beneficial effects of regular physical activity on endothelium function, progenitor cell regulation, and neovascularization to long-term recovery following mild brain ischemia. Our findings may have clinical implications for rehabilitation as there is growing interest in environmental influence on functional recovery after brain ischemia.

We have previously demonstrated that regular physical activity improves short-term stroke outcome via upregulation of eNOS. In addition to endothelium function, eNOS is essential for the functional activity of progenitor cells and the formation of new vessels. In support of this notion, voluntary running increases EPCs and improves neovascularization via NO-dependent mechanisms. Moreover, running not only increased eNOS expression in the vasculature but also within EPCs. Aicher et al have demonstrated that progenitor cell mobilization from BM is impaired in eNOS⁻/⁻ mice, and the authors have provided evidence that eNOS expression within the BM is essential for mobilization of stem and progenitor cells. To decipher how important EPC eNOS is in the exercise response, it would be interesting to perform BM transplantation with eNOS⁻/⁻ cells.

Brain ischemia induces the formation of new blood vessels that typically have altered morphology and microvascular structure. In this study, brain ischemia per se induced a BM progenitor cell response with higher number of circulating EPCs and also a proliferative response in the brain. However, in sedentary mice, this angiogenic response was apparently abortive. (1) The engraftment of TIE2/LacZ⁺ BM-derived cells was scarcely increased in the ischemic brain; (2) brain ischemia per se did not confer increased neovascularization in the disc model; (3) by 4 weeks, vessels in the ischemic territory had altered microvascular structure with grossly increased vessel caliber; and (4) absolute rCBF within the ischemic lesion remained compromised. In runners, expression of eNOS and number of EPCs in BM, blood, and spleen were upregulated compared with sedentary mice. Importantly, EPC upregulation was further enhanced by brain ischemia (Figure 8). Along with eNOS augmentation, VEGF serum levels were elevated. Increases in serum VEGF in running mice were completely abolished by cotreatment with L-NAME, which indicates that increased NO bioavailability stimulates VEGF expression. Moreover, running augmented the engraftment of TIE2/LacZ⁺ BM cells in the ischemic brain.

Figure 8. Scheme showing the relevant eNOS-dependent pathways involved in long-term stroke protection by physical activity (for details, see text).
and by 4 weeks, the number of newly generated vessel-associated cells in the ischemic territory was elevated.

After running, the density of perfused microvessels was significantly increased compared with sedentary mice, and vessel morphology was indistinguishable from sham. In contrast, in vehicle-treated ischemic animals, microvascular structures were grossly enlarged. In particular, there was a decrease of vessels <12 μm, along with an increase of those >20 and 50 μm. Moreover, whereas ACZ increased vessel caliber in running mice, it failed to do so in the ischemic striatum of sedentary mice, indicative of compromised cerebrovascular response.18 Importantly, the effects of running on microvessel density and caliber had apparent consequences for tissue perfusion: rCBF was significantly higher in the ischemic territory of runners compared with sedentary mice. Notably, the CBF values are lower than reported for awake mice but compare well compared with sedentary mice. Notably, the CBF values are lower than reported for awake mice but compare well.

Along with enhanced angiogenesis and increased CBF, we observed reduced lesion size in the postischemic brains of runners and improved functional outcome. It is unlikely that the long-term protective effects of physical activity can solely be explained by effects on CBF during brain ischemia. (1) We did not observe significant CBF differences in the striatum of runners versus control mice during MCAo; and (2) the protective effects of running on ischemic lesion size were completely abolished with L-NAME or endostatin administered after the insult. Similarly, animals deficient in eNOS were not protected by physical activity, which is in contrast to wild-type animals on the same genetic background. Taken together, we identify enhanced NO bioavailability, neovascularization, and increased blood flow as essential mechanisms of action by which regular physical activity enhances repair in the postischemic brain.

Stem and progenitor cells may contribute to vascular repair and new vessel formation.8–13,16,24 The exact role of BM-derived progenitor cells in vascular growth in the adult, however, is still a matter of controversial debate. Although some authors demonstrated vasculogenesis in the ischemic brain, there is also evidence suggesting that BM-derived cells do not differentiate into endothelial cells in the brain.12,13,25-26 Rather, TIE2-expressing BM-derived cells may turn into pericytes, which express angiogenic factors such as VEGF, fibroblast growth factor-2, and transforming growth factor-β.27 Here, we demonstrate that physical activity increases circulating EPCs and enhances the engraftment of TIE2/LacZ + BM-derived cells into vascular structures in the ischemic brain.

The protective effects of regular physical activity on brain function are well recognized, as indicated in the Roman aphorism mens sana in corpore sano (“a healthy mind in a healthy body”). Clearly, physical activity exerts pleiotropic protective effects far beyond purely vascular mechanisms. For example, voluntary exercise increases axonal regeneration and enhances neurogenesis, long-term potentiation, and learning.28,29 Both insulin-like growth factor (IGF)-1 and brain-derived neurotrophic factor (BDNF) have been implicated as downstream mediators of the neuroprotective actions of exercise.28,29 The latter can, at least in part, be explained by the fact that eNOS regulates BDNF expression in the brain. After all, voluntary running on wheels may resemble natural conditions more closely than the artificial sedentary lifestyle of laboratory mice, which in fact may represent an experimental intervention associated with impaired regeneration.

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Disclosures

None.

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