Exercise Training Reduces Neointimal Growth and Stabilizes Vascular Lesions Developing After Injury in Apolipoprotein E–Deficient Mice

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Background—Population-based studies have shown that regular physical exercise reduces cardiovascular morbidity and mortality. However, it is unknown whether these effects are solely a result of risk factor modification or whether exercise directly affects the homeostasis of the vessel wall.

Methods and Results—We subjected 19-week-old apolipoprotein E (apoE)–knockout mice (apoE<sup>−/−</sup>; n=25) to a 6-week training program on a motorized treadmill. The control group consisted of 17 sedentary mice. After 3 weeks in the program, training and sedentary mice underwent carotid artery injury with ferric chloride. Training was then resumed for another 3 weeks. Exercise did not change body weight or lipid levels in apoE<sup>−/−</sup> mice but resulted in upregulated expression of nitric oxide synthase in the endothelium. Physical training did not significantly affect the thrombotic response to injury. However, morphometric analysis of vessels harvested 3 weeks after injury showed that neointima formation was reduced in the exercising group. This resulted in a lower intima/media ratio (0.29±0.03 versus 0.41±0.03 in sedentary mice; P=0.008) and less luminal stenosis (21±2.7% versus 33±2.3%; P=0.003). Importantly, exercise reduced the number of Mac-3–positive, oxidized LDL-containing macrophages in the vessel wall while increasing the content in collagen fibers (14.1±0.9% versus 4.8±0.8%; P<0.001). Plasminogen activator inhibitor-1, tissue factor, and fibrinogen were all significantly reduced in the lesions of trained mice.

Conclusions—In the apoE<sup>−/−</sup> mouse, exercise training reduces neointimal growth and stabilizes vascular lesions after injury. These effects appear to be at least partly independent of changes in lipid levels or the initial thrombotic response to injury. (Circulation. 2004;109:386-392.)

Key Words: exercise • atherosclerosis • plaque • cholesterol • thrombosis

Data from large population-based studies indicate that regular physical exercise reduces the incidence and prevents the complications of coronary heart disease. Existing clinical evidence suggests that the beneficial effects of exercise on cardiovascular risk may be at least in part a result of a modification of various risk factors, including a reduction in obesity, improved distribution of body fat, increased sensitivity to insulin, an improved lipid profile, and lowering of blood pressure. However, it is postulated that the association between physical activity and the reduction in cardiovascular mortality may involve additional mechanisms and in particular that exercise may directly affect the homeostasis of the vessel wall.

Atherosclerosis is an inflammatory reaction to endothelial injury that is regulated by the complex interplay of adhesion molecules, cytokines, growth factors, proteases, and protease inhibitors. In the present study, we used the apolipoprotein E–knockout (apoE<sup>−/−</sup>) mouse, a particularly useful model for analyzing the pathomechanisms of atherosclerosis in vivo, to investigate how exercise training modulates vascular remodeling and lesion growth after arterial injury. Carotid artery injury was induced in apoE<sup>−/−</sup> mice with ferric chloride, a treatment previously shown to result in lesions that exhibit several histological characteristics of human atherosclerotic plaques. Our findings reveal that physical training significantly reduces neointimal size and the severity of luminal stenosis after injury, and they also suggest that exercise may stabilize the vascular lesions developing in mice under these conditions. Importantly, these favorable effects are not associated with a reduction in body weight or with changes in cholesterol levels of apoE<sup>−/−</sup> mice. These results highlight the pleiotropic effects of physical training on vascular biology and may help improve our understanding of how physical activity contributes to the prevention of death and disability from cardiovascular disease.

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Methods

Experimental Animals
Mice deficient for apolipoprotein E (apoE<sup>−/−</sup>) were obtained from Jackson Laboratories (Bar Harbor, ME). They had been backcrossed to the C57BL/6J genetic background for at least 10 generations. After weaning at the age of 3 to 4 weeks, all mice were fed regular rodent chow (Ssniff, Soest). Mice were maintained on a 12/12-hour light-dark cycle and given unlimited access to food and water. All animal care and experimental procedures were approved by the Georg August University of Goettingen and complied with national guidelines for the care and use of laboratory animals.

Training Protocol
To assess the effects of a 6-week exercise training program (1 week of acclimatization followed by 5 weeks of training) on vascular remodeling after carotid artery injury, apoE<sup>−/−</sup> mice (19 weeks of age) were randomly assigned to a training group (n=25) and a sedentary control group (n=17). Mice assigned to the training group were taught to run on a motorized rodent treadmill with shock-plate incentive (Exer-6 mol/L, Open Treadmill, Columbus Instruments) 5 days a week. To allow for acclimatization, exercise duration and speed were gradually increased during the first week, beginning with a 10-minute training period at a speed of 10 m/min on day 1 and ending with a 50-minute training period at a speed of 14 m/min on day 5. The slope of the treadmill was kept constant at 5°. During weeks 2 to 6, mice were trained for 60 minutes, 5 days a week, at a speed of 15 m/min with 2-minute rest intervals every 15 minutes. Except for the exercise period in the training group, all mice were confined to their cages throughout the study.

At the beginning of week 4, training and sedentary mice were anesthetized and underwent carotid artery injury as described below. After surgery, mice were allowed to recover for 2 days. In the training group, exercise was gradually resumed on the third (2×15 minutes) and fourth (3×15 minutes) postoperative day, and then continued according to the above protocol until day 21 after induction of injury. At this time, mice from both groups were deeply anesthetized, and the injured vessels were harvested as explained below.

Carotid Injury and Thrombosis
Mice from both groups underwent injury of the left carotid artery with 10% ferric chloride according to an established protocol. Carotid blood flow was monitored before and during a 30-minute interval after injury with an ultrasound flow probe (0.5 VB, Transonic Systems) interfaced with a flowmeter (T106, Transonic Systems) and a data acquisition program (WinDaq Lite, DATAQ Instruments).

Body Weight and Metabolic Plasma Parameters
Mice were weighed on the first day of each week during the training program and on completion of the study (at the time of euthanasia). Blood was collected from deeply anesthetized mice by cardiac puncture in 3.8% sodium citrate (final dilution). Plasma was obtained by centrifugation at 3000 rpm for 5 minutes. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were determined enzymatically (EPOS Analyzer 5060, Boehringer Mannheim).

Morphometric and Immunohistochemical Analysis
For quantitative morphometric analysis, paraffin sections were stained with Verhoeff’s elastic stain. The neointima/media ratio, and the degree of luminal stenosis were determined (ImagePro Plus; Media Cybernetics). Five sections equally spaced throughout the injured arterial segment (at 200-μm intervals) were evaluated, and the results were averaged for each animal. Mean±SEM values were calculated from 20 mice in the training group and 17 mice in the control (sedentary) group.

Antitibodies to human fibrinogen, PAI-1, and tissue factor were identified as described previously. To detect oxidized LDL, we used an antibody against murine malondialdehyde-conjugated LDL (kind gift of Dr W. Palinski, University of California at San Diego; dilution, 1:50) and an avidin-biotin phosphatase detection system (Universal APAAP kit; Dako). Endothelial nitric oxide synthase (eNOS) was localized by use of a polyclonal rabbit anti-mouse NO-3 antibody (Santa Cruz; dilution, 1:75). PAI-1 was identified with a polyclonal rabbit anti-mouse PAI-1 antibody (Santa Cruz; dilution, 1:20), and tissue factor was observed by use of a goat anti-human tissue factor antibody (American Diagnostica; dilution, 1:50). Finally, fibrinogen was detected with a rabbit anti-human fibrinogen antibody (Dako; dilution, 1:200).

Statistical Analysis
Continuous variables are presented as mean±SEM. Differences between the training and the control groups were examined by use of the Student t test for independent means. Changes in body weight between the beginning and the end of the training period were tested by the Student t test for paired means. Qualitative variables were tested by the Fisher exact test.

Results

Exercise Increases eNOS Expression but Does Not Affect the Thrombotic Response to Arterial Injury
Regular physical exercise over a period of 6 weeks did not significantly affect body weight or plasma lipid parameters in apoE<sup>−/−</sup> mice (Table). Carotid artery injury and thrombosis were induced in mice after they had been trained for 3 weeks as well as in age-matched sedentary (control) mice. At that time, mice in the training group exhibited an upregulated expression of eNOS in endothelial cells of (uninjured) carotid vessels compared with their sedentary counterparts (Figure 1). After injury with ferric chloride, complete thrombotic occlusion occurred in 18 of 25 mice (72%) in the training group compared with 14 of 17 mice (82%) in the control group (P=NS). In the vessels that occluded, the mean time to thrombotic occlusion was slightly prolonged in training compared with sedentary mice (15.6±1.2 versus 14.2±1.1 minute; P=NS). At the end of the 30-minute monitoring
period after injury, Doppler flow studies showed that injured vessels were patent in 10 of 25 mice (40%) in the training group compared with 8 of 17 mice (47%) in the control group (√P=NS).

**Exercise Reduces Neointima Formation and Luminal Stenosis**

To assess the effects of physical exercise on neointima formation and luminal stenosis 3 weeks after injury, serial sections through the injured carotid segment were stained with Verhoeff’s elastic stain and analyzed morphometrically. Figure 2 shows representative cross sections from injured vessels in the sedentary group (A and C) and the training group (B and D). Figure 3 summarizes the results of the quantitative morphometric analysis. Mice in the training group exhibited significantly reduced neointima formation after injury compared with sedentary mice (Figure 3A). In contrast to the effects on the neointima, the medial area was not significantly different between the 2 groups (data not shown). As a result, the intima/media ratio was significantly lower in training than sedentary mice (0.29±0.03 versus 0.41±0.03, √P=0.008; Figure 3B). Furthermore, luminal stenosis was significantly reduced in the training group compared with the controls (21±2.7% versus 32.5±2.3%, √P=0.003; Figure 3C).

**Exercise Alters the Composition of Arterial Lesions After Injury**

Histochemical analysis of the arterial lesions 3 weeks after injury revealed striking differences in the cellular composi-

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**Figure 1.** Physical training is associated with upregulated expression of eNOS in carotid vessels. Representative findings of immunohistochemical analysis performed on uninjured carotid arteries from sedentary mice (A), as opposed to those subjected to 3 weeks of daily physical training (B). Arrows in B denote eNOS-positive endothelial cells. Magnification ×1000.

**Figure 2.** Exercise reduces neointimal growth in apoE⁻/⁻ mice after arterial injury with ferric chloride: histological analysis. Displayed are representative sections of carotid vessels harvested 3 weeks after injury and stained with Verhoeff’s stain to facilitate visualization of elastic fibers. Arrows denote internal elastic lamina with neointima above it and 2 medial layers below. Left (A and C), control (sedentary) group; right (B and D), training group. Magnification: A and B, ×200; C and D, ×400.

**Figure 3.** Exercise reduces neointima area (A), intima/media (I/M) ratio (B), and degree of luminal stenosis (C) in apoE⁻/⁻ mice after arterial injury with ferric chloride: quantitative morphometric analysis. Five sections equally spaced throughout injured arterial segment (at 200-μm intervals) were evaluated, and results were averaged for each animal. Mean±SEM values were calculated from 17 mice in control (sedentary) group (open bars) and 20 mice in training group (solid bars).
tion between the training and control groups. For example, physical exercise resulted in a significant reduction in the Mac-3–immunopositive area, suggesting a reduction in the number of Mac-3–positive macrophages in the vessel wall (Figure 4, A versus B; quantitative analysis in C). Because most of these cells stained positive for oxLDL, exercise was also associated with a significant reduction in the oxLDL-positive area (Figure 4, D versus E; analysis in F).

Further immunohistochemical studies revealed that exercise significantly reduced the protein expression of the antifibrinolytic factor PAI-1 (Figure 5, A versus B; quantitative analysis in C) and of the procoagulant tissue factor (Figure 5, D versus E; analysis in F) in the neointima and media of arterial lesions developing in mice after injury. In addition, mice in the training group exhibited a significant reduction in intramural fibrinogen content compared with sedentary mice (Figure 5, G versus H; analysis in I). Finally, physical training was associated with a slight increase in the SMC content and, particularly, a significant increase in the collagen content of arterial lesions 3 weeks after injury (Figure 6).

Discussion

The importance of exercise and physical activity for the prevention and treatment of atherosclerotic cardiovascular disease is now widely acknowledged. Apart from the well-documented favorable effects of regular exercise on cardiovascular risk factors, it has been suggested that the benefits of physical training may involve additional mechanisms such as modulation of plasma fibrinolysis, platelet activity, and endothelium-dependent vasodilatation. In the present study, we tested the hypothesis that exercise directly affects the homeostasis of the vessel wall and modulates vascular remodeling. Using the ferric chloride model of arterial injury, we demonstrated that a 6-week training program reduced neointimal growth and luminal stenosis in hyperlipidemic apoE⁻/⁻ mice without affecting body weight or systemic cholesterol levels in these animals. Importantly, the effects of physical training on the vascular wound-healing response to injury included a reduction in the local expression of prothrombotic and antifibrinolytic factors and lower numbers of lipid-laden macrophages with a concomitant increase in collagen content. These results suggest that lifestyle modification focusing on regular exercise may directly reduce the size and increase the stability of vascular lesions developing in response to injury.

Atherosclerosis is a chronic inflammatory process initiated by endothelial injury. Favorable effects on the size and composition of atherosclerotic lesions have been observed after dietary or statin-induced lipid lowering in a rabbit model, and current guidelines emphasize the importance of lowering systemic non-HDL cholesterol to halt the progression of atherosclerosis and prevent cardiovascular disease and stroke. Although exercise may improve the lipoprotein profile in humans, lipid levels, including HDL cholesterol levels, were not significantly different between trained and sedentary apoE⁻/⁻ mice in our study and in a previous report. It thus appears that, at least in the mouse, the beneficial effects of exercise on vascular remodeling involve other mechanisms in addition to modification of the systemic lipid profile.

Treatment of mouse carotid vessels with ferric chloride reproducibly results in arterial thrombosis, which is followed...
by thrombus organization and neointima development. Considering that thrombosis and fibrin accumulation may contribute to neo-intimal formation by a variety of mechanisms and that regular physical activity may attenuate thrombosis in humans by reducing platelet aggregation and PAI-1 activity, the reduced lesion size observed in the training group of apoE−/− mice might have been expected to result from reduced thrombus formation in these animals after arterial injury. However, the possible effects of exercise on thrombosis do not suffice to explain the findings of our study. In fact, although exercise appeared to (slightly) attenuate the thrombotic response of apoE−/− mice to ferric chloride–induced injury, this effect did not reach statistical significance.

The results presented above suggest that other mechanisms in addition to those related to thrombosis may be responsible for the beneficial effects of exercise on vascular remodeling. Indeed, we could show that exercise training had a profound effect on endothelial activation and on inflammatory activity. For example, the number of lipid-laden macrophages and the expression of PAI-1, tissue factor, and fibrinogen in the arterial lesions were all significantly reduced in mice belonging to the training group. These changes were accompanied by an increase in collagen and, to a lesser extent, SMC content. All of these findings are indicative of reduced inflammatory activity and lesion stabilization and thus support the notion that the beneficial effects of physical exercise may be mediated, at least in part, by modulation of inflammation. In support of this hypothesis, clinical studies recently showed that physical activity and fitness are associated with a reduction of systemic inflammatory markers in humans.

Although the exact mechanisms mediating the anti-inflammatory effects of exercise remain to be determined, accumulating evidence points to modulation of NO synthesis as an important pathway. NO, synthesized by eNOS, may help prevent the detrimental consequences of arterial injury on the vascular wall by inhibiting platelet adhesion, macro-

Figure 5. Expression of thrombogenic factors and fibrinogen is less pronounced in vascular lesions from mice in training compared with sedentary group. Immunohistochemical staining for PAI-1 (A and B), tissue factor (D and E), and fibrinogen (G and H). Arrows denote external elastic lamina. Magnification ×400. For quantitative assessment and comparison between training and sedentary group, immunoreactive area for PAI-1, tissue factor, and fibrinogen was calculated as percentage of total vessel wall area within external elastic lamina, ie, excluding periadventitial inflammatory tissue. Results are displayed in C, F, and I, respectively. Bars represent mean±SEM of measurements performed in 6 mice per group.
phage chemotaxis, and vascular SMC migration and proliferation.\textsuperscript{21,22} In the present study, immunohistochemical analysis of uninjured vessels from apoE\textsuperscript{-/-} mice revealed upregulation of eNOS expression in the training compared with the sedentary group. This histological observation is in agreement with previous reports showing that physical exercise increases the availability of endothelium-derived NO.\textsuperscript{23} In fact, the results of recent studies suggest that modulation of NO production by exercise may limit atherosclerotic plaque size and, possibly, intimal hyperplasia after balloon angioplasty.\textsuperscript{21,24}

Finally, it needs to be mentioned that experimental models of arterial injury, including the ferric chloride model used in the present study, cannot exactly reproduce the pathophysiology of human atherosclerosis, atherothrombosis, or restenosis after angioplasty and stenting. Notwithstanding this important limitation, lesions developing in mice after injury with ferric chloride exhibit several histological characteristics of human atherosclerotic plaques,\textsuperscript{8,9} and their systematic study helps dissect basic pathomechanisms of the vascular wound healing (remodeling) process in the presence of cardiovascular risk factors such as hyperlipidemia.\textsuperscript{11}

In conclusion, our studies using the ferric chloride model of arterial injury in the apoE\textsuperscript{-/-} mouse demonstrate that exercise training exerts pleiotropic favorable effects on the vascular response to injury that are at least partly independent of risk factor modification. These results may help explain the clinical benefits of exercise in both cardiovascular disease prevention and cardiac rehabilitation.

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