

Regular Physical Activity Improves Endothelial Function in Patients With Coronary Artery Disease by Increasing Phosphorylation of Endothelial Nitric Oxide Synthase

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Background—In stable coronary artery disease (CAD), exercise training has well-documented positive effects on arterial endothelial function. NO derived from endothelial NO synthase (eNOS) is regarded as a protective factor against atherosclerosis. The aim of the present study was to investigate the effects of exercise training on the endothelial function in relation to the expression of eNOS and Akt-dependent eNOS phosphorylation in the left internal mammary artery (LIMA) of patients with stable CAD.

Methods and Results—In 17 training patients (T) and 18 control patients (C), endothelium-dependent vasodilation and average peak flow velocity (APV) in response to acetylcholine were measured invasively at study beginning and after 4 weeks in the LIMA. In LIMA tissue sampled during bypass surgery, eNOS expression and content of phospho-eNOS-Ser¹¹⁷⁷, Akt, and phospho-Akt were determined by Western blot and quantitative reverse transcriptase–polymerase chain reaction. After exercise training, LIMA APV in response to acetylcholine was increased by $56 \pm 8\%$ (from $+48 \pm 8\%$ at beginning to $+104 \pm 11\%$ after 4 weeks, $P < 0.001$). Patients in T had a 2-fold higher eNOS protein expression (T 1.0 ± 0.7 versus C 0.5 ± 0.3 arbitrary units, $P < 0.05$) and 4-fold higher eNOS Ser¹¹⁷⁷-phosphorylation levels in LIMA-endothelium (1.2 ± 0.9 versus 0.3 ± 0.2 arbitrary units, $P < 0.01$). A linear correlation was confirmed between Akt phosphorylation and phospho-eNOS levels ($R = 0.80$, $P < 0.05$) and between phospho-eNOS and Δ APV ($R = 0.59$, $P < 0.05$).

Conclusions—Exercise training in stable CAD leads to an improved agonist-mediated endothelium-dependent vasodilatory capacity. The change in acetylcholine-induced vasodilatation was closely related to a shear stress-induced/Akt-dependent phosphorylation of eNOS on Ser¹¹⁷⁷. (*Circulation*. 2003;107:3152-3158.)

Key Words: coronary disease ■ endothelium ■ exercise ■ nitric oxide synthase

In stable coronary artery disease, endothelial dysfunction plays a major role in the pathogenesis of exercise-induced angina pectoris. Whereas normal coronary arteries dilate, atherosclerotic coronary arteries often exhibit a paradoxical vasoconstriction in response to flow or acetylcholine, thereby causing critical ischemia even in moderate epicardial stenosis.¹

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Chronic intermittent increases of shear stress associated with exercise training, however, have been shown to improve coronary endothelial function and to reduce pathological vasoconstriction.² Because of difficulties in harvesting human arterial tissue samples, the molecular mechanisms mediating these beneficial training effects in the human circulation are still unclear.

Studies using cultured endothelial cells^{3,4} and animal experiments⁵ suggest that increases in endothelial NO synthase (eNOS) expression and protein phosphorylation are possible mechanisms. Several groups have reported that eNOS is phosphorylated at position Ser¹¹⁷⁷ (human sequence) in endothelial cells subjected to fluid shear stress by the serine/threonine protein kinase Akt^{3,6} or protein kinase A,⁷ resulting in increased activity and consequently NO production.

To critically assess the relevance of these regulatory pathways in patients with stable CAD, we randomized patients with coronary multivessel disease scheduled for elective aortocoronary bypass grafting (CABG) to either 4 weeks of in-hospital regular aerobic exercise training or to an inactive control group and quantified eNOS expression and phosphorylation in left internal mammary artery (LIMA) samples harvested during CABG.

Received January 10, 2003; revision received April 3, 2003; accepted April 4, 2003.

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Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000074229.93804.5C

Methods

Patients

Thirty-five male patients ≤ 70 years old with stable CAD were studied. Patients were eligible for the study if they had a preserved left ventricular function (left ventricular ejection fraction $\geq 60\%$), a physical work capacity ≥ 50 W, and an indication for an elective bypass surgery using the LIMA as graft. Exclusion criteria were concomitant diseases effecting endothelial function, including untreated hypertension, smoking, hypercholesterolemia, diabetes mellitus (type 1 and 2), recent myocardial infarction (within the last 4 weeks), and significant stenosis of the left main coronary artery. The protocol of this study was approved by the ethics committee of the University of Leipzig, and written informed consent was obtained from all patients before enrollment into the study.

Study Design

Patients scheduled for elective bypass surgery were randomized either into a training or an inactive control group. Before randomization and after 4 weeks, an invasive measurement of the endothelial function was performed in the LIMA. At the time of bypass surgery, approximately 36 hours after the last training session and 24 hours after the invasive measurement of endothelial function, a small piece of the LIMA was obtained and immediately frozen in liquid nitrogen for subsequent molecular analysis.

Exercise Training Program

Patients of the training group exercised in hospital under supervision of a physician 3 times daily for 10 minutes on row ergometer and 3 times daily for 10 minutes on bicycle ergometer (in addition to 5 minutes for warming up and 5 minutes for cooling down) for 4 weeks. Workloads were adjusted so that patients did not experience chest pain and did not show any signs of ischemia in the ECG during exercise.

Patients assigned to the control group continued their sedentary lifestyle, resumed treatment with their individually tailored cardiac medication, and were supervised by their private physicians.

In Vivo Measurement of Endothelial Function

In vivo endothelial function was assessed in the LIMA using the same drug concentrations and applying the same technique as previously described in detail for coronary arteries.² Treatment with any cardiovascular medication was discontinued for at least 24 hours before measurement of LIMA endothelial function. At baseline, patients were given 10 000 U of heparin, and a 6F guiding catheter was used to cannulate the LIMA.

Organ Chamber Experiments

Organ chamber experiments were performed using phenylephrine-precontracted LIMA rings.⁸ After 30 minutes of equilibration in Krebs buffer, the maximal constriction was achieved by adding KCl to a final concentration of 100 mmol/L to the Krebs buffer. After rinsing the ring carefully several times, the LIMA was precontracted to 60% of maximal KCl constriction by adding increasing concentrations of phenylephrine (Sigma; 10^{-9} to 10^{-3} mol/L). Relaxations to acetylcholine (10^{-9} to 10^{-4} mol/L; Sigma) or sodium nitroprusside (10^{-10} to 10^{-4} mol/L; Sigma) were recorded. The EC_{50} was defined as concentration necessary to achieve a 50% LIMA ring relaxation.

Immunohistochemical Staining for CD31

The expression of CD31 was visualized on paraffin sections using a monoclonal anti-CD31 (PECAM-1) antibody (clone JC/70A; Dako Diagnostika GmbH; dilution 1:50) and the alkaline-phosphatase anti-alkaline phosphatase method according to the manufacturer's instruction.

RNA Isolation and Quantification of mRNA Expression

Quantitative RT-PCR was performed with total RNA⁹ using the Light Cycler system (Roche Diagnostics Inc). The following primers

TABLE 1. Clinical Parameters of Study Population

	Training Group (n=17)	Control Group (n=18)
Age, y	64 \pm 4	65 \pm 8
LVEF, %	63 \pm 8	66 \pm 8
LVEDD, mm	50 \pm 4	50 \pm 8
Previous MI, n	7	7
No. of affected coronary vessels (2/3), n	6/11	7/11
BMI, kg/m ²	28.0 \pm 2.8	28.0 \pm 2.9
Serum glucose, mmol/L	5.8 \pm 1.2	5.9 \pm 0.8

LVEF indicates left ventricular ejection fraction; LVEDD, left ventricular end diastolic diameter; MI, myocardial infarction; and BMI, body mass index. Data presented are mean \pm SD or total number.

No significant difference for all parameters between the 2 groups was detected.

were used: eNOS: 5'-GTGTTTGCCGAGTCCTCACC-3' and 5'-CTCCTGCAAGGAAAAGCTCTG-3'; von Willebrand factor: 5'-TGCTGACACCAGAAAAGTGC-3' and 5'-AGTCCCAATGGA-CTCACAG-3'.

Quantification of Protein Expression

Frozen tissue samples were homogenized in lysis buffer,¹⁰ and Western blot analysis was performed as described previously.⁹ To specifically detect total eNOS, p-eNOS-Ser¹¹⁷⁷, total-Akt, and p-Akt-Ser⁴⁷³ commercially available antibodies (Calbiochem; New England Biolabs) were applied. In a first step, p-eNOS and p-AKT was detected on the same membrane. After stripping, the blot was reprobbed with antibodies recognizing total eNOS and total Akt. To control for loading differences, the blots were also reprobbed with an antibody against GAPDH (Hyttest, Turku, Finland). All samples were analyzed in triplicate.

Statistical Analysis

All measurements and analysis of all measures were done in a blinded fashion. Data are expressed as mean \pm SD. Comparisons between the training and the control group were performed with an unpaired *t* test or a 2-way repeated-measures ANOVA, followed by a Tukey post hoc test, where appropriate. Linear regression analysis was applied to determine the relationship between phospho-Akt, phospho-eNOS, and change in APV, diameter, and blood flow, respectively. $P < 0.05$ was considered statistically significant.

Results

Baseline Characteristics

Thirty-five patients were randomly assigned to the exercise training group (n=17) or the control group (n=18). The 2 groups did not differ significantly with respect to the baseline parameters (Table 1). Most patients were taking β -receptor antagonist (88% in the training group and 94% in the control group), angiotensin-converting enzyme inhibitors (65% and 50%), β -HMG-CoA reductase inhibitors (88% and 94%), aspirin (100% and 94%), and nitrates (41% and 56%).

Clinical Follow-Up

Two patients in the training and 1 patient in the control group refused the invasive reassessment of endothelial function at 4 weeks. After exercise training, no significant change of clinical characteristics (left ventricular ejection fraction, left ventricular end-diastolic diameter, and body mass index) were detected, and metabolic variables in the training group remained essentially unchanged (total cholesterol: beginning,

TABLE 2. Effect of Exercise Training on Vessel Diameter

Drug and Dose	% Change in Vessel Diameter vs Baseline			
	Exercise Training Group (n=17)		Control Group (n=18)	
	Study Beginning	Follow-Up at 4 Weeks	Study Beginning	Follow-Up at 4 Weeks
ACH 0.072 μ g/min	2.1 \pm 3.7	4.4 \pm 3.7*†	1.7 \pm 2.5	1.7 \pm 3.4
ACH 0.72 μ g/min	4.8 \pm 4.5	9.3 \pm 5.3*†	4.6 \pm 3.8	4.9 \pm 3.4
ACH 7.2 μ g/min	6.1 \pm 6.1	13.2 \pm 6.6*†	7.3 \pm 5.0	7.3 \pm 3.8
Adenosine 2.4 mg/min	7.0 \pm 2.9	12.1 \pm 5.3†	9.2 \pm 5.4	9.8 \pm 4.6
Nitroglycerin 200 μ g	10.0 \pm 6.1	9.0 \pm 4.5	10.7 \pm 7.1	11.3 \pm 5.9

ACH indicates acetylcholine.

* P <0.05 vs control group, † P <0.05 vs beginning.

4.71 \pm 0.82 versus 4.69 \pm 1.1 mmol/L after 4 weeks, P =NS; LDL: beginning 3.03 \pm 0.73 versus 2.92 \pm 0.77 mmol/L after 4 weeks, P =NS; HDL: beginning 1.12 \pm 0.24 versus 1.13 \pm 0.20 mmol/L after 4 weeks, P =NS; triglycerides: beginning 1.70 \pm 0.77 versus 1.52 \pm 0.86 mmol/L after 4 weeks, P =NS).

In Vivo Measurement of Endothelial Function

Endothelium-Dependent Vasodilation

At study beginning, patients in the exercise training and control group had similar responses to acetylcholine, expressed as percentage change from baseline luminal diameter (infusion of normal saline) (Table 2). After 4 weeks of exercise training, the mean vasodilatory response to the ascending concentrations of acetylcholine (0.07, 0.72, and 7.2 μ g/min) was significantly increased by 109% (from 0.06 \pm 0.08 to 0.12 \pm 0.12 mm; P =0.076 versus beginning, P <0.05 versus control), by 94% (from 0.14 \pm 0.12 to 0.27 \pm 0.12 mm; P <0.05 versus beginning and control), and by 116% (from 0.18 \pm 0.16 to 0.38 \pm 0.16 mm; P <0.05 versus beginning and control), whereas no significant change was detectable in patients of the control group (Table 2).

After exercise training, acetylcholine led to a significantly greater increase in LIMA mean peak blood flow velocity (Δ APV) compared with baseline. In the exercise training group, the increase versus baseline was 767% (from 0.6 \pm 3.6 to 4.6 \pm 6.9 cm/s; P <0.05 versus beginning and control) with

acetylcholine at a dosage of 0.072 μ g per minute, 194% (from 4.9 \pm 5.3 to 9.5 \pm 9.4 cm/s; P <0.05 versus beginning, P =0.063 versus control) at a dosage of 0.72 μ g per minute, and 197% (from 11.6 \pm 7.4 to 22.8 \pm 11.8 cm/s; P <0.05 versus beginning) at a dosage of 7.2 μ g per minute (Table 3). However, in the control group, no significant change in Δ APV was noticed between the initial study and the follow-up at 4 weeks (Table 3).

Endothelium-Independent Vasodilation

The vasodilatory reaction of the LIMA in response to the endothelium-independent vasodilator nitroglycerin (200 μ g as a bolus) remained basically unchanged after 4 weeks of exercise training (an increase of 0.29 \pm 0.16 mm in luminal LIMA diameter at the initial study versus an increase of 0.26 \pm 0.16 after 4 weeks; P =NS). The maximal increase in LIMA mean peak blood flow velocity caused by nitroglycerin was 207.8 \pm 20.6% at the initial study and 189.2 \pm 14.1% after 4 weeks of exercise training (P =NS). The difference was not statistically significant, and the response to nitroglycerin in the control group was similar (Tables 2 and 3).

Flow-Dependent Dilatation

Exercise training resulted in a significant increase in flow-dependent dilatation (assessed by adenosine infusion; 2.4 mg/min) of the proximal LIMA by 150% (from 0.18 \pm 0.08 to 0.27 \pm 0.12 mm, P <0.05 versus beginning, P =0.10 versus control). In contrast, in the control group, adenosine-induced

TABLE 3. Effect of Exercise Training on Mean Peak Blood Flow Velocity (APV)

Drug and Dose	% Change in Mean Peak Blood Flow Velocity vs Baseline			
	Exercise Training Group (n=17)		Control Group (n=18)	
	Study Beginning	Follow-Up at 4 Weeks	Study Beginning	Follow-Up at 4 Weeks
ACH 0.072 μ g/min	3.2 \pm 15.1	24.1 \pm 34.4*†	-2.8 \pm 7.9	-5.7 \pm 11.3
ACH 0.72 μ g/min	21.1 \pm 21.7	47.7 \pm 46.3*†	16.9 \pm 15.9	16.7 \pm 18.4
ACH 7.2 μ g/min	47.9 \pm 31.1	104.3 \pm 45.9*†	53.0 \pm 30.2	61.6 \pm 48.7
Adenosine 2.4 mg/min	202.0 \pm 74.6	239.3 \pm 96.7*†	163.7 \pm 97.4	152.7 \pm 84.0
Nitroglycerin 200 μ g	207.8 \pm 84.4	189.2 \pm 57.8	161.5 \pm 94.0	162.1 \pm 87.4

ACH indicates acetylcholine.

* P <0.05 vs control group; † P <0.05 vs beginning.

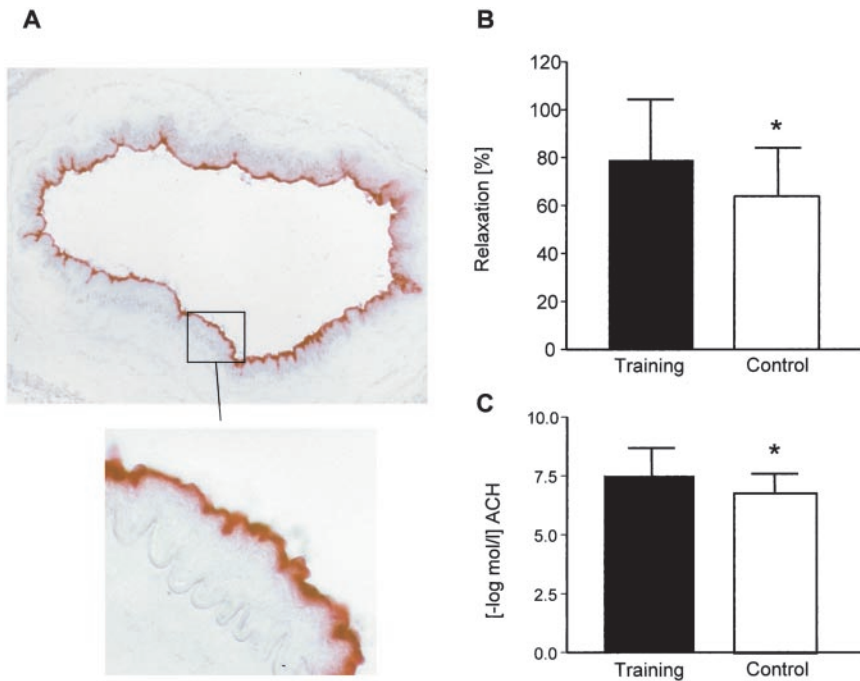


Figure 1. CD31 immunohistochemistry and in vitro measurement of acetylcholine-mediated vasodilatation in LIMA rings. The endothelial cell layer is stained red, confirming an intact endothelium in the LIMA rings. The concentration of acetylcholine to induce a maximal (B) and 50% relaxation (C) was calculated for both groups.

changes in proximal LIMA diameter at 4 weeks did not differ significantly from the values at study beginning.

In Vitro Measurement of Endothelial Function

To confirm the presence of the endothelial cell layer, the expression of CD31 was visualized in the rings by immunohistochemistry after the in vitro experiments. As shown in Figure 1A, the endothelial cell layer is intact, lining the whole vessel.

Measurements of endothelium-dependent and -independent LIMA vasodilatation were repeated in the tissue bath to study endothelial function in a standardized environment not affected, for example, by increased circulating levels of oxidative stress. Exposing LIMA rings to increasing concentrations of acetylcholine resulted in a significantly better maximal ring relaxation in the training group compared with the control group (Figure 1B). To induce 50% relaxation of phenylephrine-precontracted LIMA rings, a significantly lower concentration of acetylcholine was necessary in the training group compared with the control group (Figure 1C).

Using increasing concentrations of sodium nitroprusside, whose action is endothelium-independent, no difference was observed between the 2 groups (maximal relaxation in the control group, $97 \pm 2\%$; in the training group, $95 \pm 3\%$).

Expression of eNOS and peNOS-Ser¹¹⁷⁷

In the exercise training group, the eNOS mRNA expression in the LIMA was 96% higher compared with the control group (T 6.11 ± 4.5 versus C 3.12 ± 2.4 arbitrary units; $P < 0.05$) (Figure 2A). This exercise-induced augmentation of eNOS-mRNA content was accompanied by a doubling of eNOS protein in the training group (T 1.03 ± 0.71 versus C 0.56 ± 0.30 arbitrary units; $P < 0.05$) (Figure 2B). Using antibodies specifically directed against the Ser¹¹⁷⁷ phosphorylation site of the eNOS, a basal phosphorylation was detectable

in the LIMA of the control group (C 0.28 ± 0.24 arbitrary units). However, patients in the training group showed a 300% higher eNOS phosphorylation (T 1.18 ± 0.93 arbitrary units; $P < 0.05$, Figure 2C). Moreover, the ratio of phosphorylated to unphosphorylated eNOS was 3-fold higher in the exercise training group compared with the control group (T 1.82 ± 1.95 versus C 0.63 ± 0.57 ; $P < 0.05$), indicating that a higher proportion of eNOS is phosphorylated than newly generated under the influence of exercise training (Figure 2D).

Expression of Akt and Akt Phosphorylation Status

To evaluate the influence of regular aerobic exercise training on the activation of Akt, the amount of total Akt and Ser⁴⁷³-pAkt was determined. The expression of total Akt in the training group (0.70 ± 0.57 arbitrary units) did not differ significantly from that in the control group (0.87 ± 0.40 arbitrary units). In contrast, the level of Akt phosphorylation at Ser⁴⁷³ was elevated by 90% in the training group (T 1.31 ± 1.01 versus C 0.69 ± 0.42 arbitrary units; $P < 0.05$) (Figure 3A). Analyzing the ratio of Ser⁴⁷³-pAkt to total Akt, an even higher—nearly 3.5-fold—increase was apparent in the LIMA of patients after exercise training compared with the LIMA of sedentary controls (T 2.67 ± 2.17 versus C 0.83 ± 0.61 ; $P < 0.05$; Figure 3B).

To investigate whether in the LIMA the phosphorylation state of Akt accounts for the phosphorylation of eNOS on Ser¹¹⁷⁷, a correlation analysis between both parameters was performed in the training group. The linear relation between Ser⁴⁷³-pAkt and Ser¹¹⁷⁷-peNOS ($r = 0.80$, $P < 0.05$) underlines that this signaling mechanism is substantial and still functioning in patients with CAD (Figure 4A).

Impact of eNOS Phosphorylation on Endothelial Function of the LIMA

To determine whether the phosphorylation of eNOS on Ser¹¹⁷⁷ has a physiological effect on endothelium-dependent vasodi-

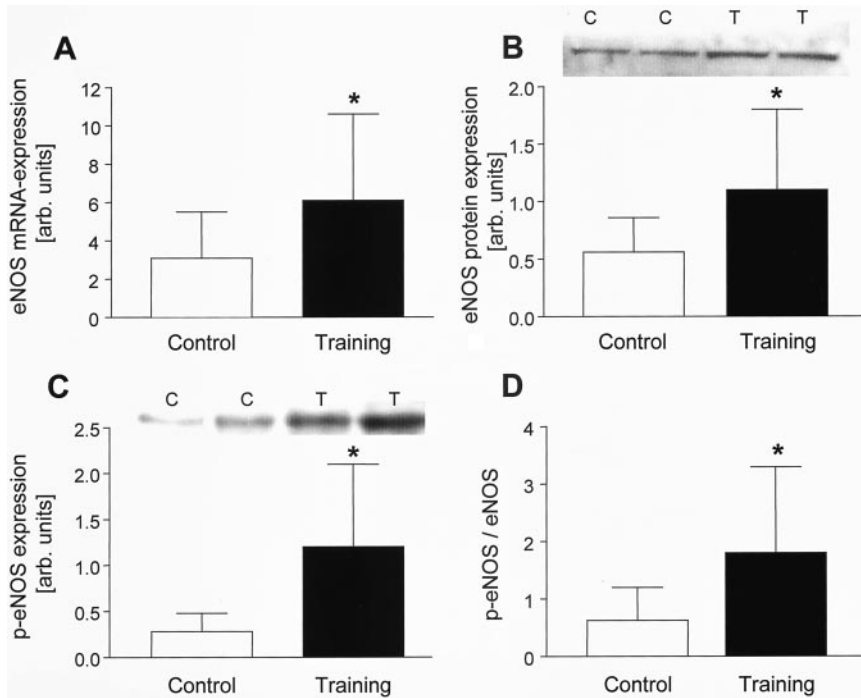


Figure 2. Influence of exercise training on the mRNA and protein expression of eNOS and Ser¹¹⁷⁷-phosphorylated eNOS in the LIMA of patients with CAD. The mRNA expression (A) in the LIMA of the training and control group was determined by quantitative RT-PCR and expressed as ratio to von Willebrand factor. To determine the total protein content of eNOS (B) and the Ser¹¹⁷⁷ phosphorylated form of eNOS (C), Western blot analysis was performed. On top of the figures, representative Western blots of the training (T) and the control group (C) are depicted. The ratio of phosphorylated versus unphosphorylated eNOS is significantly increased (D) under the influence of exercise training. Results are expressed as mean \pm SD. * $P < 0.05$ vs control.

lation, we performed a correlation analysis in the training group involving eNOS phosphorylation and Δ APV (difference between the change in APV from baseline at the highest dosage of acetylcholine in the initial study and at 4 weeks). A linear relation between p-eNOS-Ser¹¹⁷⁷ and Δ APV was observed ($r = 0.59$, $P < 0.05$; Figure 4B).

Discussion

Analyzing regulatory pathways by which exercise training improves agonist-mediated vasodilation in patients with stable CAD, the LIMA was used as a target vessel assessable for both in vivo function analysis and ex vivo molecular studies in patients scheduled for elective CABG. Three important messages emerge from this study. First, exercise training improves agonist-mediated endothelium-dependent vasodilatory capacity of the LIMA in patients with CAD. Second, this is accompanied by an increased eNOS protein expression and most likely Akt-dependent eNOS phosphorylation at Ser¹¹⁷⁷. Third, the state of eNOS phosphorylation at Ser¹¹⁷⁷ correlates with the improvement of endothelial function in vivo.

Improvement of Endothelial Function

CAD is characterized by systemic endothelial dysfunction, not only of atherosclerotic vessels but also of vessels usually not prone to atherosclerosis, like the radial artery or the LIMA.^{8,11} Different attempts have been made to correct the impaired endothelium-dependent vasodilatation.^{12,13} Apart from pharmacological interventions, exercise training has been shown to improve endothelium-dependent vasodilatation in coronary conduit and resistance vessels of patients with CAD.² In the present study, we found that 4 weeks of aerobic exercise training improved LIMA endothelial function in patients with symptomatic stable CAD measured in vivo and in vitro. In vivo, the vasodilatation and the APV of the LIMA in response to acetylcholine were significantly enhanced after exercise training. In addition, the adenosine-induced flow-dependent vasodilatation of the LIMA after exercise training was markedly improved. These in vivo results were confirmed by in vitro experiments of isolated LIMA rings. In the organ bath, rings from training patients showed an improved relaxation in response to acetylcholine.

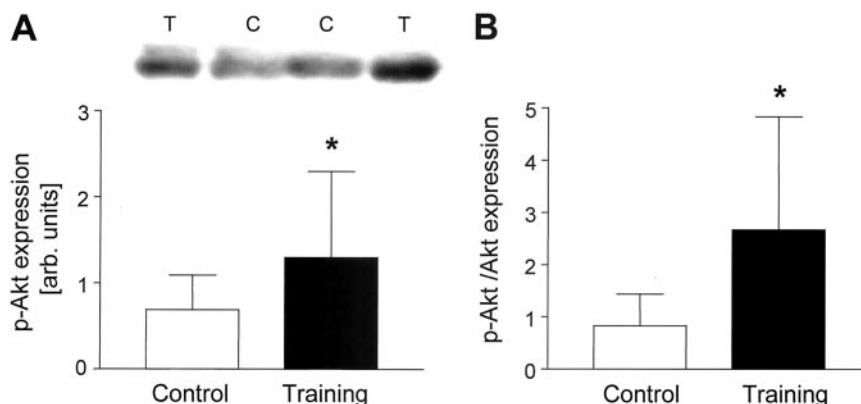


Figure 3. Influence of exercise training on the phosphorylation of Akt at Ser⁴⁷³ in the LIMA of patients with CAD. The protein content of Ser⁴⁷³-pAkt was determined by Western blot analysis and quantified by densitometry (A). On top of the panels, representative Western blots of the training (T) and the control group (C) are shown. Analyzing the ratio of Ser⁴⁷³-pAkt to total-Akt (B), a highly significant increase was obvious under the training conditions. Results are expressed as the mean \pm SD. * $P < 0.05$ vs control.

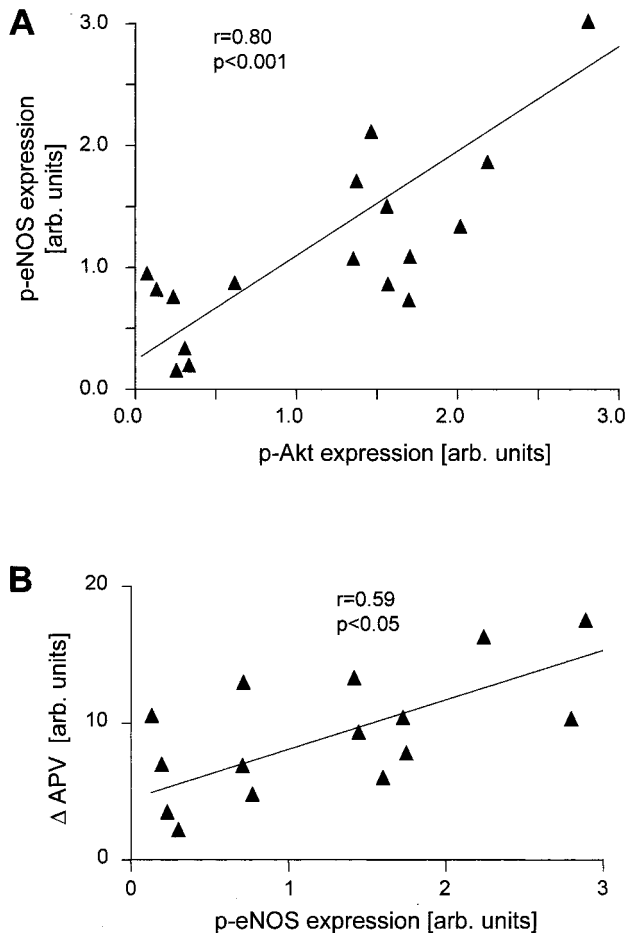


Figure 4. Relation between the amount of phosphorylated Akt and the amount of Ser¹¹⁷⁷ phosphorylated eNOS (A) in the LIMA of the training group and the impact of Ser¹¹⁷⁷ phosphorylation of eNOS endothelial function (B). A linear correlation between the amount of Ser¹¹⁷⁷ phosphorylated eNOS after 4 weeks and the percentage change in APV (4 weeks vs study beginning) in the training group is depicted.

The dilatation capacity of the rings investigated in the present study is comparable with a recent report investigating the endothelium-dependent vasoactivity of the LIMA in different diseases.⁸ For ethical reasons, harvesting LIMA from patients without CAD was not possible. Therefore, we are unable to confirm the presence of pathological endothelial function based on *in vitro* data. However, analyzing the flow-dependent vasodilatation in the radial artery, a vessel also not prone to atherosclerosis, a significant impairment in patients with CAD could be detected compared with healthy controls.¹⁴ Based on these observations, it is very likely that also in the LIMA the agonist-mediated endothelium-dependent vasodilatation is significantly impaired compared with healthy individuals.

In contrast, nitroglycerin-induced, endothelium-independent LIMA vasodilatation did not differ significantly between the exercise training and the control group. This indicates that the response of the vascular smooth muscle to NO was not impaired. Furthermore, it confirms previous studies showing no effect of exercise training on endothelium-independent vasodilatation despite a remarkable im-

provement of agonist-mediated endothelium-dependent vasodilatation in patients with CAD.²

Regulation of eNOS Expression

The current concept of shear stress-induced changes in eNOS expression/activity is based on cell culture experiments or animal models: In both human umbilical vein endothelial cells and bovine arterial endothelial cells, a significant increase in eNOS expression was observed after 6 hours of exposure to laminar shear stress.^{15–17} Furthermore, it was confirmed in animal models that exercise training had effects similar to *in vitro* shear stress on eNOS expression in coronary conduit⁵ and resistance vessels.¹⁸

The present study is the first one confirming the training effects on vascular function and eNOS expression in the human vascular system. We found a 2-fold increase in eNOS mRNA expression and vascular protein content after only 4 weeks of regular physical exercise training. The rise in eNOS expression is probably mediated by shear stress-responsive elements in the promoter region of the eNOS gene¹⁹ or by stabilization of eNOS mRNA.¹⁷ The exact mechanism by which the endothelial cell senses changes in flow or blood pressure amplitude, however, is still widely unknown.

Besides eNOS protein expression, NO production also depends on eNOS phosphorylation. Among the 5 potential eNOS phosphorylation sites, the Ser¹¹⁷⁷ residue seems to function as a sensor of shear stress, because exposure of endothelial cells to laminar shear stress specifically increases the phosphorylation at this site.²⁰ This leads to a rise in the enzymatic activity of eNOS and enhanced NO production.^{3,20} Interestingly, the shear stress-induced phosphorylation of eNOS is maintained,³ whereas the agonist-mediated Ser¹¹⁷⁷ phosphorylation, eg, by bradykinin, is only transient.²¹ This indicates that exercise training leads to an improved endothelial function, probably by a sustained eNOS phosphorylation, resulting in an increased NO production or availability.

We found a 300% higher eNOS phosphorylation on Ser¹¹⁷⁷ in patients with CAD after 4 weeks of daily aerobic exercise training compared with sedentary controls. Therefore, exercise training seems to have a dual positive effect in those patients: it increases the protein expression of eNOS, which may take a couple of hours, and it enhances the phosphorylation of eNOS at Ser¹¹⁷⁷ in minutes after application of shear stress,^{6,20} thereby leading to an increased vascular NO production. However, exercise training seems to have a higher impact on the phosphorylation of eNOS on Ser¹¹⁷⁷, because it was increased 3.2-fold in the exercise training group whereas protein expression was only doubled compared with the inactive controls. Besides the phosphorylation of eNOS on Ser¹¹⁷⁷, other potential phosphorylation sites, like Thr⁴⁹⁵ and Ser¹¹⁶, have been reported in the current literature.^{20,22} Especially in agonist-mediated activation of eNOS, the dephosphorylation of Thr⁴⁹⁵ plays an important role in the regulation of eNOS activity.^{21,22} Nevertheless, the application of fluid shear stress to endothelial cells in culture only altered the phosphorylation status of Ser¹¹⁷⁷.^{6,20}

What are possible intracellular signaling events leading to shear stress-induced phosphorylation of eNOS-Ser¹¹⁷⁷? Cell culture studies using pharmacological inhibitors^{6,20} suggest

that activation of PI3K leads to an activation/phosphorylation of the protein kinase Akt, which phosphorylates eNOS at the residue Ser¹¹⁷⁷.

This hypothesis is additionally supported by a study by Luo et al²³ quantifying the vasomotor activity of rabbit femoral arteries transduced with a constitutively active Akt. In these vessels, they found a significant increase in resting vessel diameter and blood flow compared with those transduced with a dominant-negative Akt construct. The authors concluded that Akt, possibly via the phosphorylation of eNOS and an increased NO bioavailability, functions as a key regulator of vascular tone in vivo. Because ethical reasons preclude the usage of specific inhibitors of Akt and inducible NOS phosphorylation, we were unable to determine the exact signaling pathway in vivo. However, the relevance of the above-proposed signaling pathway on the basis of cell culture experiments is supported by the close correlation between p-Akt and p-eNOS in patients with CAD (Figure 4A).

Association Between eNOS Phosphorylation and Endothelial Function

Recently, the significance of eNOS-Ser¹¹⁷⁷ phosphorylation on acetylcholine-mediated vessel dilatation was investigated in eNOS knockout animals by Scotland et al.²⁴ They could clearly demonstrate that animals transduced with a nonphosphorylatable eNOS gene had a significantly reduced acetylcholine-mediated vasodilatation compared with animals transduced with an eNOS phosphorylatable at Ser¹¹⁷⁷. Based on this finding, one might speculate that the exercise training-induced upregulation of the eNOS protein expression together with the enhanced phosphorylation leads to an improvement of endothelial function. The correlation between the proportion of eNOS phosphorylated on Ser¹¹⁷⁷ and the change of endothelium-dependent blood flow induced by exercise training is consistent with the hypothesis that the increase in eNOS protein expression and phosphorylation leads to an improvement of endothelium function in patients with CAD.

In summary, exercise training has beneficial effects on endothelial dysfunction in symptomatic patients with CAD. At the cellular level, this beneficial training effect is closely related to a shear stress-induced/Akt-dependent increase in eNOS phosphorylation on Ser¹¹⁷⁷.

Acknowledgments

This study was supported by grant HA 2165/4-1 from the Deutsche Forschungsgemeinschaft. The authors thank Silke Krabbes, Jeanine Böger, and Angela Kricke for excellent technical assistance.

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