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## Impact of Regular Physical Activity on the NAD(P)H Oxidase and Angiotensin Receptor System in Patients With Coronary Artery Disease

Volker Adams, PhD; Axel Linke, MD; Nicolle Kränkel, PhD; Sandra Erbs, MD; Stephan Gielen, MD; Sven Möbius-Winkler, MD; Jan F. Gummert, MD; Friedrich Wilhelm Mohr, MD; Gerhard Schuler, MD; Rainer Hambrecht, MD

**Background**—In patients with stable coronary artery disease, physical exercise training (ET) improves endothelial dysfunction. A potential mechanism mediating the enhanced vasomotor function is a reduced breakdown of endothelium-derived nitric oxide by reactive oxygen species (ROS). The aim of the present study was to analyze the impact of ET on sources of ROS generation in the left internal mammary artery of patients with symptomatic coronary artery disease.

**Methods and Results**—In left internal mammary artery rings sampled during bypass surgery from 45 patients randomized to either a training (n=22) or an inactive control (n=23) group, the mRNA expression of NAD(P)H oxidase subunits, NAD(P)H oxidase activity, and ROS production were assessed. In addition, endothelial function, expression of angiotensin II (Ang II) receptor type 1 and 2 (AT<sub>1</sub>-R and AT<sub>2</sub>-R), and Ang II-mediated vasoconstriction were determined. ET resulted in a significant lower expression of gp91<sup>phox</sup> (23.1±0.5 versus 69.1±18.1 arbitrary units, training versus control), p22<sup>phox</sup> (0.7±0.3 versus 2.0±0.5 arbitrary units), and Nox4 (2.7±1.2 versus 5.4±1.0 arbitrary units). Enzymatic activity (2.1±0.3 versus 4.9±0.4 mU/mg) and ROS generation (0.02±0.01 versus 0.06±0.02 arbitrary units) were significantly lower in the training compared with the control group. On a functional level, ET resulted in improved acetylcholine-mediated vasodilatation and a 49% reduction in Ang II-induced vasoconstriction, accompanied by lower AT<sub>1</sub>-R (3.7±0.8 versus 16.6±5.7 arbitrary units, training versus control) and higher AT<sub>2</sub>-R (7.8±2.5 versus 1.6±0.7 arbitrary units) mRNA expression.

**Conclusions**—ET reduces vascular expression of NAD(P)H oxidase and AT<sub>1</sub>-R, resulting in decreased local ROS generation. These molecular effects converge in a reduced Ang II-mediated vasoconstriction. (*Circulation*. 2005;111:555-562.)

**Key Words:** angiotensin ■ endothelium ■ exercise ■ polymerase chain reaction ■ vessels

Activation of the renin-angiotensin-aldosterone system occurs in the context of both normal cardiovascular aging and numerous cardiovascular diseases, including hypertension, chronic heart failure, and coronary artery disease (CAD).<sup>1,2</sup> In the vascular wall, angiotensin II (Ang II) leads to a direct vasoconstrictive response through activation of the predominant Ang II type 1 receptor (AT<sub>1</sub>-R) of vascular smooth muscle cells. In addition, Ang II has been shown to induce NAD(P)H oxidase activity and to increase local ROS production. Consequently, the half-life of endothelium-derived nitric oxide (NO) is significantly shortened by increased ONOO<sup>-</sup> generation in the presence of reactive oxygen species (ROS).<sup>3</sup>

NAD(P)H oxidase is a multienzyme complex composed of the membrane-associated glycoprotein gp91<sup>phox</sup> (also known

as Nox2), p22<sup>phox</sup>, and 3 cytosolic components: p40<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup>.<sup>4,5</sup> Recently, 4 homologues of gp91<sup>phox</sup>/Nox2 called Nox1, Nox3, Nox4, and Nox5 have been identified.<sup>6,7</sup> In vascular tissue, mainly Nox1, Nox2, and Nox4 are expressed in endothelial and vascular smooth muscle cells.<sup>8,9</sup> At the cellular level, NAD(P)H oxidases are known to be activated by mechanical forces and hormones, particularly Ang II,<sup>10</sup> and cytokines.<sup>1</sup> In vascular smooth muscle cells, the binding of Ang II to the AT<sub>1</sub>-R leads to a phosphorylation of p47<sup>phox</sup>, initiating the translocation of this subunit to the cell membrane and assembly of the enzyme complex.<sup>11</sup> Moreover, Ang II increases the expression of all NAD(P)H oxidase subunits.<sup>12</sup> The Ang II-induced elevation in oxidative stress is counteracted by the activation of the Ang II type 2 receptor (AT<sub>2</sub>-R), and the net effect of Ang II

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on vasomotion depends mainly on the local ratio of AT<sub>1</sub>-R to AT<sub>2</sub>-R expression on the cell surface.<sup>13</sup>

Regular physical exercise training has recently been shown to improve endothelium-dependent coronary vasodilatation.<sup>14</sup> On the molecular level, training has been shown to enhance vascular NO production by increasing endothelial NO synthase (eNOS) expression and by shear stress-mediated Akt-dependent eNOS phosphorylation<sup>15</sup>; however, changes in ROS-related NO degradation might also be involved. We therefore hypothesized that exercise training would reduce local vascular oxidative stress by reducing Ang II-induced NAD(P)H oxidase activation.

## Methods

### Patients

Forty-five male patients  $\leq 70$  years of age with stable CAD were studied. Patients were eligible for the study if they had a preserved left ventricular function ( $\geq 60\%$ ), a physical work capacity of  $\geq 50$  W, and an indication for an elective bypass surgery with the left internal mammary artery (LIMA) as the bypass graft. Concomitant diseases affecting endothelial function (untreated hypertension, smoking, hypercholesterolemia, diabetes mellitus) or prohibiting exercise training (recent myocardial infarction within the last 4 weeks, significant stenosis of the left main coronary artery) were regarded as exclusion criteria.

During the study period, patients in both groups were encouraged to continue their nutritional habits. They did not receive special counseling.

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.

### Study Design

Patients with CAD scheduled for elective bypass surgery were randomized either to a training group or to an inactive control group. Before randomization and after 4 weeks, an invasive measurement of the endothelial function was performed in the LIMA as recently described in detail.<sup>15</sup> Patients in the training group exercised in the hospital under close supervision of a physician 3 times daily for 10 minutes on a row ergometer and 3 times daily for 10 minutes on a bicycle ergometer (plus a 5-minute warm-up and a 5-minute cool-down) for 4 weeks. Workloads were adjusted so that patients did not experience chest pain or show any signs of ischemia in the ECG during exercise. Patients assigned to the control group continued their sedentary lifestyle and treatment with their individually tailored cardiac medication, and they were supervised by their private physicians. At the time of bypass surgery, a small piece of the LIMA was obtained. One part of the sample was snap-frozen in liquid nitrogen for subsequent molecular analysis; the other was used for functional analysis of Ang II-induced vasoconstriction in an organ bath.

### RNA Isolation and Quantification of mRNA Expression

The LIMA was cleared of connective tissue, and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with total RNA<sup>16</sup> applying the Light Cycler system (Roche Diagnostics Inc). The following primers were used: gp91<sup>phox</sup>, 5'-ATCCATATCCGCATCGTTGG-3' and 5'-ATGGATGCCAAGG-GTGTGAC-3'; p22<sup>phox</sup>, 5'-CAGATCGAGTGGGCCATGTG-3' and 5'-TCCATGGTGGAGCCCTT CTT-3'; Nox1, 5'-TAACAGCACG-CTGATCCTG-3' and 5'-CTGGAGAGAATGGAG GCAAG-3'; Nox4, 5'-ACAGGGGTCTGC ATGGTGGT-3' and 5'-GCCGGG-AGGGTGGGTATCTA-3'; AT<sub>1</sub>-R, 5'-CAGTATGGAATACCGC-TGGCC-3' and 5'-ATGATGATGCAGGTGACTTTG-3'; and AT<sub>2</sub>-R, 5'-AAATATGCCCAATGGTCAGC-3' and 5'-AAGGAAGTGCCAGG TCAATG-3'. 18S rRNA was amplified as a housekeeping gene: 5'-

ATACAGGACTCTTTCGAGGCC-3' and 5'-CGGGACTCAGC-TAAGAGCAT-3'.

### Quantification of Protein Expression

Frozen tissue samples were homogenized in lysis buffer,<sup>17</sup> and Western blot analysis was performed as previously described.<sup>15</sup> To detect specific proteins, the following antibodies were applied: AT<sub>1</sub>-R and AT<sub>2</sub>-R (both Santa Cruz) and gp91<sup>phox</sup> (Biozol). To control for loading differences, the blots were also reprobed with an antibody against GAPDH (Hyttest). All samples were analyzed in triplicate.

### Quantification of Oxygen Radical Formation and NAD(P)H Oxidase Enzyme Activity

Total superoxide production was measured by superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described previously.<sup>18</sup> Briefly, a segment of the LIMA ring was homogenized and incubated with cytochrome *c* (80  $\mu\text{mol/L}$ ) for 30 minutes at 37°C in the presence or absence of PEG-SOD (40 U; Sigma), followed by measurement of absorbance at 550 nm. Superoxide production was calculated as the portion of total ferricytochrome *c* reduction that was inhibitable by SOD and normalized to tissue wet weight.

The enzymatic activity of NAD(P)H oxidase was measured photometrically in tissue homogenate to monitor the proportion of cytochrome *c* reduction at 550 nm in the presence of 100  $\mu\text{mol/L}$  NADH, which is inhibitable by SOD (60 U) and diphenylene iodonium (10  $\mu\text{mol/L}$ ). Previous experiments demonstrated that, under these assay conditions, the ROS responsible for the cytochrome *c* reduction derives predominantly from the NAD(P)H oxidase, not from other sources (see the online-only Data Supplement).

### Organ Chamber Experiments

The vessel rings were cleared of connective tissue and equilibrated for 30 minutes in Krebs' buffer before endothelial function was measured in an organ bath. To determine the maximal contractile response, potassium chloride was added to the bath at a final concentration of 100 mmol/L. After the ring was carefully rinsed several times, the LIMA was incubated in increasing concentrations of Ang II ( $10^{-10}$  to  $10^{-6}$  mol/L; Sigma). The vasoconstrictive response to different Ang II concentrations was calculated as percent of maximal KCl-induced contraction. All measurements were performed by a technician blinded to patient group assignment.

### Statistical Analysis

All measurements and analyses of all measures were done in a blinded fashion. Data are expressed as mean  $\pm$  SEM. Comparisons between the training and control groups were performed by an unpaired *t* test or a 2-way repeated-measures ANOVA followed by Tukey post hoc test when appropriate. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Baseline Characteristics

Forty-five patients were randomly assigned to the exercise training (n=22) or the control (n=23) group. The 2 groups did not differ significantly with regard to baseline parameters (see the Table). Most patients were on  $\beta$ -receptor antagonists (92% of the training group, 96% of the control group), ACE inhibitors (92% and 96%),  $\beta$ -HMG-CoA-reductase inhibitors (77% and 66%), aspirin (100% and 96%), and nitrates (35% and 50%).

The subgroup of patients in whom LIMA samples were available for NAD(P)H oxidase activity, ROS measurement, and functional analysis of Ang II-induced vasoconstriction

**Clinical Parameters of the Study Population**

	Training Group (n=22)	Control Group (n=23)
Age, y	63±2	63±2
LVEF, %	62±2	65±2
LVEDD, mm	49±1	50±1
Previous MI, n	10	9
Affected coronary vessels (2/3), n	8/14	10/13
BMI, kg/m <sup>2</sup>	27.9±0.8	27.9±0.5
Serum glucose, mmol/L	5.7±0.3	5.8±0.2

LVEF indicates left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; MI, myocardial infarction; and BMI, body mass index.

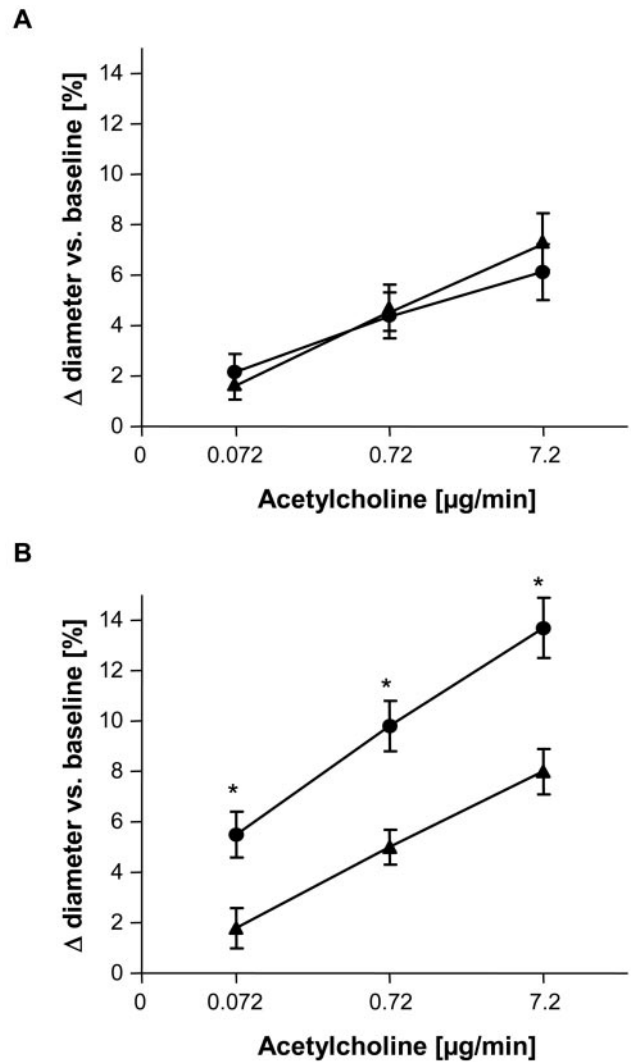
(n=14) did not differ from the entire group (n=45) with regard to baseline parameters (age, 61±3 versus 63±2 years in the entire study group, *P*=NS; left ventricular ejection fraction, 61±2% versus 64±2% in the entire study group, *P*=NS) and medication (data not shown).

**Clinical Follow-Up**

Exercise training did not lead to a significant change in left ventricular ejection fraction, left ventricular end-diastolic diameter, body mass index, and metabolic variables in the training group (total cholesterol, 4.64±0.17 mmol/L at the beginning versus 4.50±0.27 mmol/L after 4 weeks, *P*=NS; LDL, 2.91±0.15 versus 3.00±0.18 mmol/L, *P*=NS; HDL, 1.10±0.05 versus 1.14±0.04 mmol/L, *P*=NS; triglycerides, 1.71±0.15 versus 1.95±0.22 mmol/L, *P*=NS). During the 4-week study period, no changes were observed in the control group either (data not shown). A significant reduction in blood pressure was noted in the training group (RR<sub>sys</sub>, 125.5±3.5 versus 110.4±2.4 mm Hg, *P*<0.05; RR<sub>dia</sub>, 77.6±2.4 versus 69.6±2.3 mm Hg, *P*<0.05), whereas no significant change was observed in the control group (RR<sub>sys</sub>, 125.9±4.2 versus 128.9±3.9 mm Hg, *P*=NS; RR<sub>dia</sub>, 78.2±2.0 versus 80.3±2.0 mm Hg; *P*=NS). High-sensitivity C-reactive protein at the beginning of the study and after 4 weeks showed no significant difference between the control and training groups (beginning, 1.6±0.3 mg/L in control versus 1.5±0.3 mg/L in training, *P*=NS; after 4 weeks, 1.4±0.4 mg/L in control versus 1.2±0.3 mg/L in training, *P*=NS). In addition, the study medication and dosage were not altered during the study period in the control and training groups.

**In Vivo Measurement of Endothelial Function**

At the start of the study, patients in the exercise training and control groups had similar responses to increasing concentrations of acetylcholine, expressed as percentage change from baseline luminal diameter (infusion of normal saline) (Figure 1A). After 4 weeks of exercise training, the mean vasodilatory response to ascending concentrations of acetylcholine (0.07, 0.72, and 7.2 μg/min) was significantly increased by 160% (from 0.05±0.02 to 0.13±0.02 mm, *P*<0.05 versus beginning and control), 118% (from 0.11±0.02 to 0.24±0.03 mm, *P*<0.01 versus beginning and control), and 126% (from 0.15±0.03 to 0.34±0.04 mm, *P*<0.001 versus beginning and control), whereas no significant change was

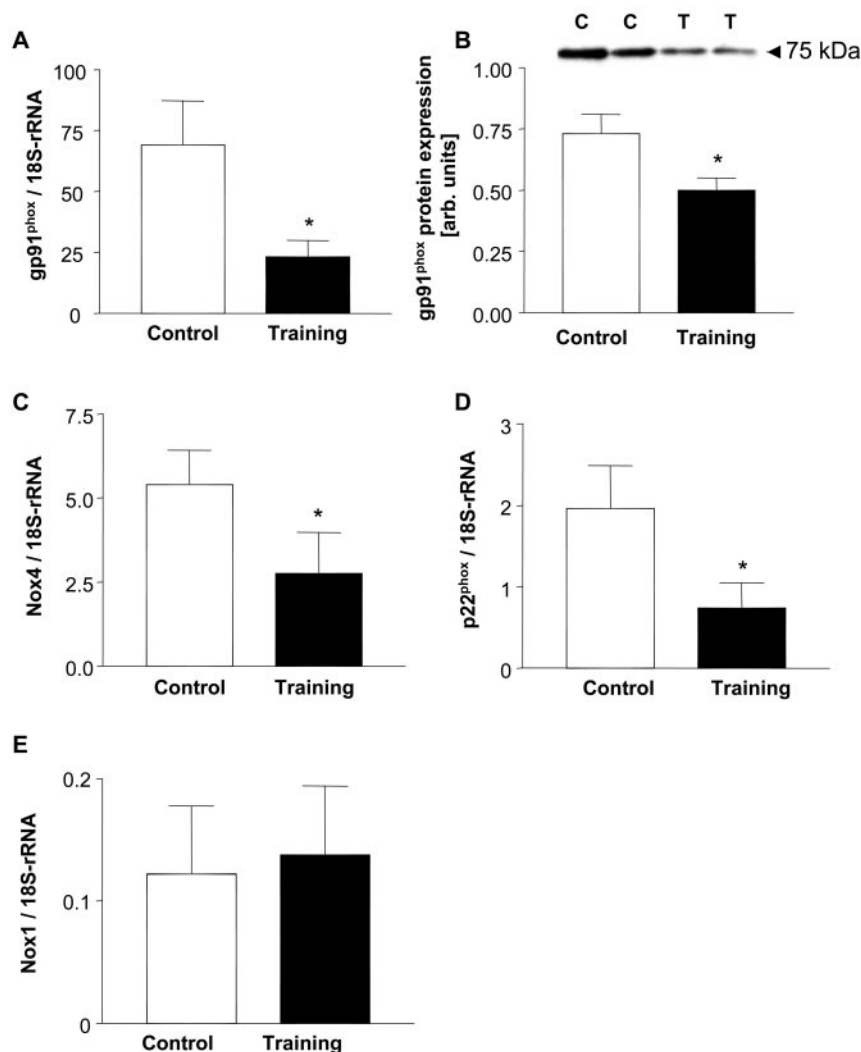


**Figure 1.** Endothelial function measured in vivo in relation to increasing concentrations of acetylcholine at study beginning (A) and after 4 weeks (B) in training (●) and control (▲) groups. Endothelial function is expressed as change in vessel diameter from baseline. \**P*<0.05 vs control.

detectable in patients in the control group (Figure 1B). The vasodilatory reaction of the LIMA in response to the endothelium-independent vasodilator nitroglycerin did not change after 4 weeks of exercise training (an increase of 0.25±0.03 mm in luminal diameter at study beginning versus 0.30±0.04 mm after 4 weeks, *P*=NS).

**Expression of NAD(P)H Oxidase Subunits**

In the exercise training group, a diminished mRNA expression of gp91<sup>phox</sup> by 67% was observed compared with the control group (23.1±6.8 versus 69.1±18.1 arbitrary units, respectively; *P*<0.005 versus control) (Figure 2A). The training-associated lower mRNA expression was linked to a 30% lower protein expression of this isoform (0.50±0.05 versus 0.73±0.08 arbitrary units; *P*<0.05 versus control) (Figure 2B). Additionally, a significantly diminished mRNA expression of Nox4 and the subunit p22<sup>phox</sup> of the NAD(P)H multienzyme complex was de-



**Figure 2.** Influence of exercise training on mRNA and protein expression of NAD(P)H oxidase subunits in LIMA of CAD patients. mRNA expression (A) and protein expression (B) of gp91<sup>phox</sup> were determined by quantitative RT-PCR and Western blot analysis. Top, Representative Western blot of training (T) and control (C) groups. Additionally, mRNA expression of Nox4 (C), p22<sup>phox</sup> (D), and Nox1 (E) was quantified and expressed as ratio to 18S-rRNA. Results are expressed as mean±SEM. \**P*<0.05 vs control.

tected (Nox4,  $2.7 \pm 1.2$  versus  $5.4 \pm 1.0$  arbitrary units, *P*<0.001 versus control; p22<sup>phox</sup>,  $0.7 \pm 0.3$  versus  $2.0 \pm 0.5$  arbitrary units, *P*<0.001 versus control) (Figure 2C and 2D). Nox1 mRNA expression did not change in response to exercise training ( $1.38 \pm 0.71$  versus  $1.22 \pm 0.56$  arbitrary units, *P*=NS) (Figure 2E).

#### NAD(P)H Oxidase Activity and Superoxide Production

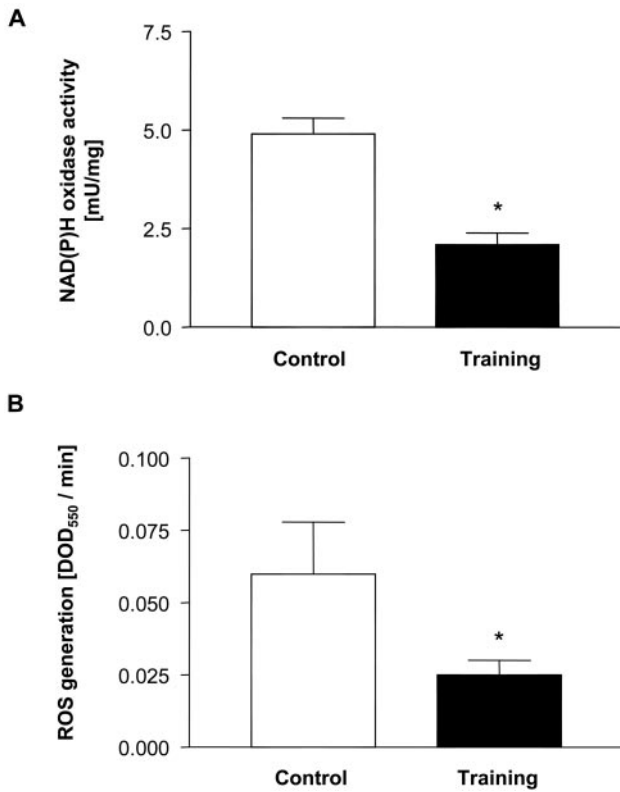
The relevance of the lower mRNA/protein expression of different NAD(P)H oxidase subunits was confirmed by measuring total NAD(P)H oxidase enzyme activity and ROS production. Because of the limited sample size, the analysis was performed in a subgroup of patients (7 from each group). The enzymatic NAD(P)H oxidase activity ( $2.1 \pm 0.3$  mU/mg for training versus  $4.9 \pm 0.4$  mU/mg for control, *P*=0.002 versus control; Figure 3A) and overall ROS production ( $0.025 \pm 0.005$  versus  $0.060 \pm 0.018$  arbitrary units, *P*<0.05 versus control; Figure 3B) were considerably attenuated in the LIMA rings of the training group. Moreover, a linear correlation between the enzymatic activity of NAD(P)H oxidase and generation of ROS could be documented ( $r=0.57$ , *P*<0.05).

#### Influence of Exercise Training on AT<sub>1</sub>-R and AT<sub>2</sub>-R Expression

Because Ang II is a major factor regulating NAD(P)H oxidase through activation of AT<sub>1</sub>-R or AT<sub>2</sub>-R, the expression of both receptor subtypes was analyzed in the LIMA of the training and control groups. At the mRNA level, AT<sub>1</sub>-R expression was 77% lower in the training group ( $3.7 \pm 0.8$  versus  $16.6 \pm 5.7$  arbitrary units, *P*<0.05 versus control) (Figure 4A), whereas the AT<sub>2</sub>-R expression was ≈5-fold higher ( $7.75 \pm 2.46$  versus  $1.58 \pm 0.68$  arbitrary units, *P*<0.05 versus control) (Figure 4B). The reduction in AT<sub>1</sub>-R mRNA expression was associated with a 46% lower protein expression as analyzed by Western blot analysis ( $0.47 \pm 0.09$  versus  $0.87 \pm 0.12$  arbitrary units for training versus control, *P*=0.008) (Figure 4C); however, AT<sub>2</sub>-R protein expression was not different between groups ( $0.51 \pm 0.10$  versus  $0.53 \pm 0.12$  arbitrary units, training versus control; *P*=NS) (Figure 4D).

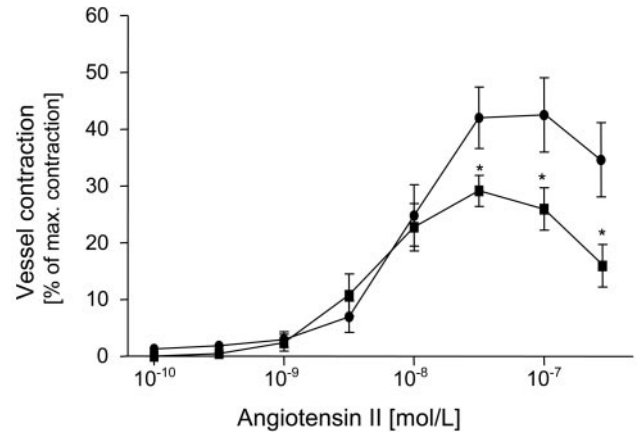
#### In Vitro Measurement of Ang II–Mediated Contraction

Ang II–induced vasomotion was analyzed in an organ bath in the same subgroup of patients. Exposure of LIMA rings to



**Figure 3.** Enzymatic activity of NAD(P)H oxidase (A) and overall production of ROS (B) were determined in homogenate of LIMA rings from patients in control and training groups. Results are expressed as mean±SEM. \**P*<0.05 vs control.

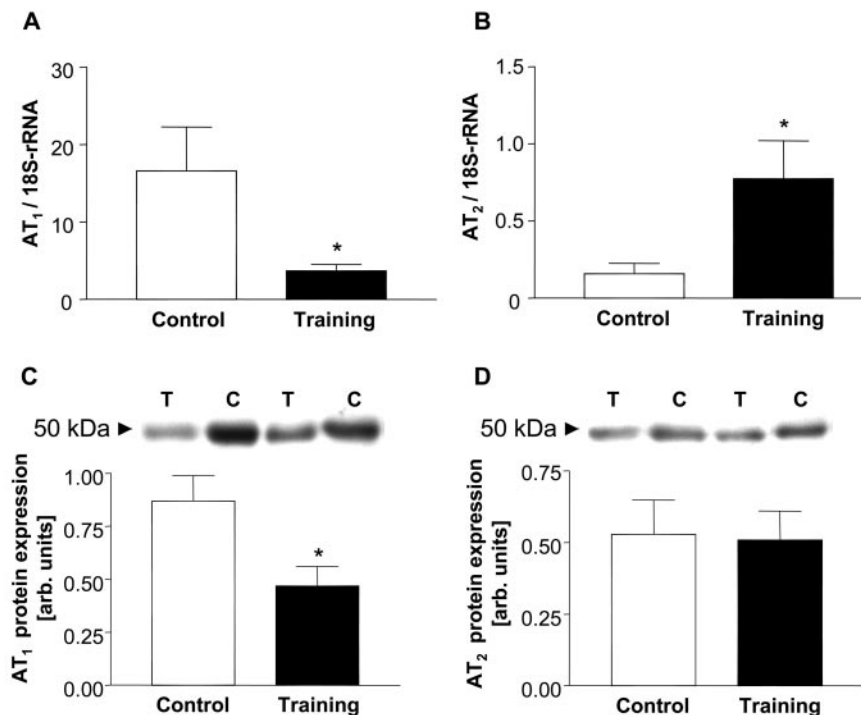
increasing concentrations of Ang II resulted in a 49% lower maximal contraction in the training group compared with the control group (23.1±6.1% versus 45.2±8.8% of maximal KCl contraction, respectively) (Figure 5).



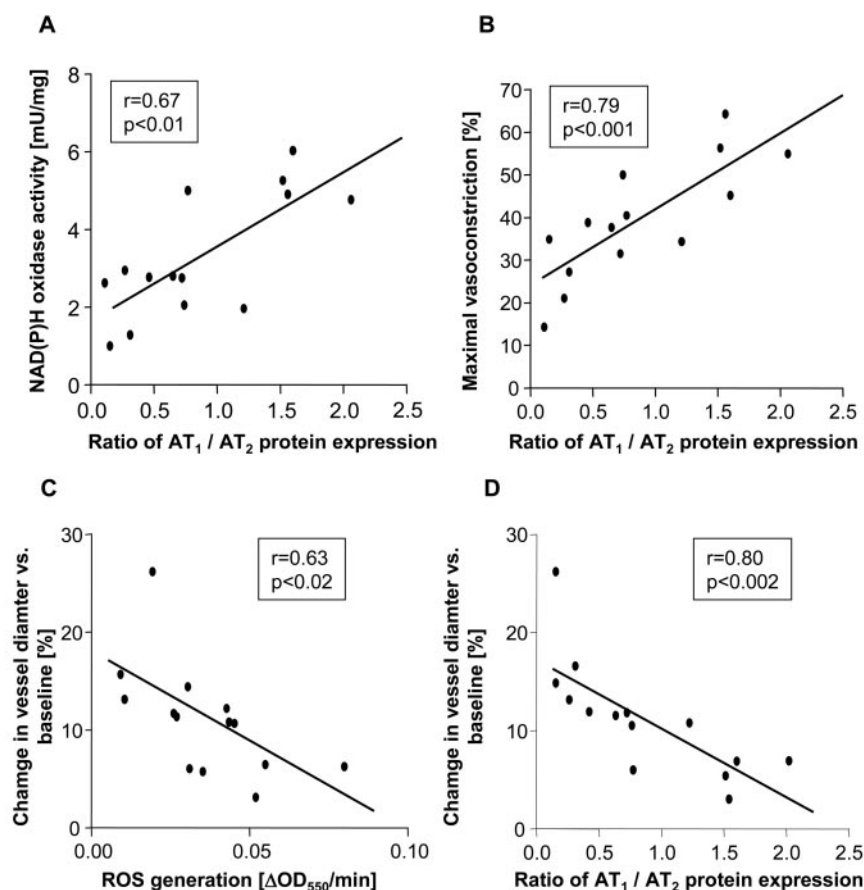
**Figure 5.** In vitro measurement of Ang II-mediated vasoconstriction in LIMA rings. Concentration curve for Ang II to induce maximal contraction was calculated for training (■) and control (●) groups. \**P*<0.05 vs control.

**Impact of the AT<sub>1</sub>/AT<sub>2</sub>-R Ratio and gp91phox Expression on Ang II-Mediated Vasomotion**

To determine whether the AT<sub>1</sub>/AT<sub>2</sub>-R ratio has an impact on NAD(P)H oxidase activity and Ang II-mediated vasomotion, a correlation analysis involving AT<sub>1</sub>/AT<sub>2</sub>-R protein expression, NAD(P)H oxidase activity, and Ang II-mediated maximal vasoconstriction was performed. A linear relation between AT<sub>1</sub>/AT<sub>2</sub> protein expression and NAD(P)H oxidase activity (*r*=0.67, *P*<0.01; Figure 6A) and between AT<sub>1</sub>/AT<sub>2</sub>-R protein expression and Ang II-mediated maximal vasoconstriction (*r*=0.79, *P*<0.001; Figure 6B) was observed. In addition, a significant linear correlation between NAD(P)H oxidase enzymatic activity and Ang II-mediated maximal vasoconstriction (*r*=0.56, *P*<0.05) and between ROS generation and Ang II-mediated maximal vasoconstriction (*r*=0.52, *P*<0.05) could be documented.



**Figure 4.** Influence of exercise training on mRNA and protein expression of AT<sub>1</sub>-R and AT<sub>2</sub>-R subtypes in LIMA of CAD patients. mRNA expression of AT<sub>1</sub>-R (A) and AT<sub>2</sub>-R (B) in LIMA of training (T) and control (C) groups was determined by quantitative RT-PCR and expressed as ratio to 18S-rRNA. To determine total protein content of AT<sub>1</sub>-R (C) and AT<sub>2</sub>-R (D), Western blot analysis was performed. Top, Representative Western blots of the training and control groups. Results are expressed as mean±SEM. \**P*<0.05 vs control.



**Figure 6.** Linear correlation between ratio of AT<sub>1</sub>/AT<sub>2</sub> expression and NAD(P)H oxidase activity (A) and ratio of AT<sub>1</sub>/AT<sub>2</sub>-R expression and maximal Ang II-mediated vasoconstriction (B). Linear correlations between endothelial function, measured as change in vessel diameter after application of acetylcholine (7.2 μg/min), ROS generation (C), and AT<sub>1</sub>/AT<sub>2</sub> expression (D) are shown.

### Impact of Biochemically Detected Alterations on Endothelial Function in the LIMA

To determine whether the AT<sub>1</sub>/AT<sub>2</sub>-R ratio, NAD(P)H oxidase activity, and ROS production affect endothelial function, we performed a correlation analysis in the subgroup of patients (n=14) in whom the molecular parameters were measured. An inverse linear relationship between the change in vessel diameter and ratio of AT<sub>1</sub>/AT<sub>2</sub> expression ( $r=-0.80$ ,  $P<0.01$ ) and between the change in vessel diameter and NAD(P)H oxidase activity ( $r=-0.74$ ,  $P<0.01$ ) and ROS production ( $r=-0.63$ ,  $P<0.02$ ) was observed.

### Discussion

Premature degradation of endothelium-derived NO by ROS is regarded as a key factor in the development of endothelial dysfunction. In this prospective randomized clinical trial, we assessed the effects of regular physical exercise on components of the Ang II-NAD(P)H oxidase system as a major source of oxidative stress in human endothelium of patients with stable CAD. Four major findings emerge from this clinical trial. First, the expression of subunits from the NAD(P)H oxidase enzyme complex and enzymatic activity are significantly reduced by physical exercise, resulting in a diminished overall production of superoxide anions. Second, at the molecular level, exercise training led to reduced mRNA and protein expression of AT<sub>1</sub>-R in the LIMA, decreasing the AT<sub>1</sub>/AT<sub>2</sub>-R ratio, which is important for the vasoactive net effect of Ang II. Third, the expression level of AT<sub>1</sub>/AT<sub>2</sub>-R

correlated with NAD(P)H oxidase activity and with the Ang II-mediated vasoconstriction of the LIMA. Finally, NAD(P)H oxidase activity and ROS production after 4 weeks correlate negatively with improved endothelial function.

Overt coronary atherosclerosis is accompanied by systemic endothelial dysfunction as a consequence of a reduced vascular NO bioavailability resulting from a disequilibrium between NO production and NO breakdown. Various potential sources of vascular ROS formation have been described, ie, increased NAD(P)H oxidase activity,<sup>13,19,20</sup> increased activity of cytochrome P450<sup>21</sup> and xanthine oxidase,<sup>22</sup> uncoupling of the NO synthase,<sup>23</sup> and decreased local antioxidative protection.<sup>24</sup> Besides cytochrome P450, NAD(P)H oxidase activation seems to be the most important source of O<sub>2</sub><sup>-</sup> production in the vascular wall.<sup>2,19,25</sup> This notion is further confirmed by our findings that the NADH-mediated cytochrome *c* reduction is inhibited by 85% in the presence of diphenylene iodonium, whereas the inhibition of xanthine oxidase, uncoupled NOS, and mitochondrial activity has nearly no impact.

### Influence of Exercise Training on NAD(P)H Oxidase and Oxidative Stress

Four weeks of regular physical exercise significantly improve endothelial function and increased shear stress-induced Akt-dependent eNOS phosphorylation in patients with CAD.<sup>15</sup> In this consecutive study, we showed that an attenuated expression and activity of the NAD(P)H oxidase occur as a

consequence of exercise training and increased NO production.<sup>15</sup> The lower enzymatic activity of the NAD(P)H oxidase in the LIMA of patients in the exercise training group is related to significantly diminished ROS generation. At the level of mRNA expression, mainly the subunits gp91<sup>phox</sup>, Nox4, and p22<sup>phox</sup> are affected by increased shear stress resulting from the training intervention. The magnitude of the training effect on gp91<sup>phox</sup> mRNA (69% compared with the control group) compares favorably with previous studies of statins in patients with CAD in whom ≈60% reduction in this NAD(P)H subunit was measured.<sup>26</sup>

Comparing the expression levels of the gp91<sup>phox</sup> homologues Nox1 and Nox4 shows that ≈50-fold higher expression of Nox4 was detected. These results are consistent with previous cell culture studies in bovine aortic endothelial cells in which pulsatile shear stress resulted in a downregulation of gp91<sup>phox</sup> and Nox4 mRNA expression and reduced ROS generation.<sup>27</sup> The higher relevance of Nox4 for the production of ROS by the NAD(P)H oxidase is supported by antisense experiments.<sup>9</sup> In the presence of antisense against Nox4, a significant reduction in O<sub>2</sub><sup>-</sup> production was observed, supporting the notion that Nox4 may function as the major catalytic component of an endothelial NAD(P)H oxidase.

In summary, our results are consistent with the hypothesis that exercise training reduces ROS formation in the vessel wall by the downregulation of NAD(P)H oxidase subunits, especially gp91<sup>phox</sup>, p22<sup>phox</sup>, and Nox4.

### Influence of Exercise Training on AT<sub>1/2</sub>-R Expression

NAD(P)H oxidase expression and activity are regulated by the dominant influence of AT<sub>1</sub>-R activation by inducing a rapid rac1 translocation to the cell membrane.<sup>4,5,10</sup> Changes in AT<sub>1</sub>-R expression could therefore play an important role in NAD(P)H oxidase activation. Other factors include hypoxia, hormones, mechanical stress, and inflammatory activation.<sup>19</sup>

As documented by RT-PCR and Western blotting, the expression of AT<sub>1</sub>-R was 50% lower in the training group compared with the inactive control group. Compared with a standard pharmacological intervention, exercise training was slightly less effective than statin treatment in hypercholesterolemic men, in whom the AT<sub>1</sub>-R density in platelets was reduced to 26% compared with levels before treatment.<sup>28</sup> On the other hand, mRNA expression of AT<sub>2</sub>-R was significantly higher after training, resulting in a lower AT<sub>1</sub>/AT<sub>2</sub>-R ratio.

Because the Ang II induction of NAD(P)H oxidase activity and subsequent generation of ROS are counteracted by the binding of Ang II to the AT<sub>2</sub>-R, the net effect of Ang II may depend mainly on the ratio of AT<sub>1</sub>/AT<sub>2</sub>-R expression on the vascular smooth muscle cell surface. This is supported by cell culture studies analyzing the Ang II-induced expression of gp91<sup>phox</sup>.<sup>29</sup> At low concentrations of Ang II (100 nmol/L), a preferential binding to only the AT<sub>1</sub>-R, a gp91<sup>phox</sup> mRNA induction, and elevated ROS production were found. At higher concentrations of Ang II (1 μmol/L) at which the AT<sub>2</sub>-R is activated, no influence on the expression level of gp91<sup>phox</sup> and ROS production was documented.

### Ang II–Mediated Vasoconstriction and Exercise Training

A similar biphasic effect of Ang II was observed during *in vitro* measurements of Ang II–mediated vasoconstriction.

This phenomenon was first described for the radial artery<sup>30</sup> in which low concentrations (<50 μmol/L Ang II) led to the expected vasoconstrictive response, whereas higher concentrations (>10 μmol/L Ang II) induced less vasoconstriction.

What are the possible mechanisms leading to the blunted vasoconstriction of Ang II after exercise training? It is well established that Ang II exerts its classic physiological effects such as vasoconstriction, aldosterone and vasopressin release, and sodium and water retention through the activation of the AT<sub>1</sub>-R. Because exercise training influences the ratio of AT<sub>1</sub>/AT<sub>2</sub>-R, one might speculate that the vasoconstrictive action of Ang II is blunted by the shift in Ang II receptor expression. This assumption is supported by the linear correlation between the AT<sub>1</sub>/AT<sub>2</sub>-R expression and the maximal Ang II–induced vasoconstriction. In addition, animal studies showed that the disruption of the AT<sub>2</sub>-R gene caused an increase in blood pressure and higher sensitivity to the vasoconstrictive action of Ang II.<sup>31,32</sup>

### Association Between the Reduction of ROS Formation and Endothelial Function

Altered endothelium-dependent vascular relaxation has been associated with enhanced degradation of NO by ROS in animal and human models of many diseases.<sup>1</sup> Using gp91<sup>phox</sup>-knockout animals, Jung and colleagues<sup>33</sup> clearly demonstrated recently that the formation of ROS by endothelial NAD(P)H oxidase accounts for the reduced NO bioavailability and subsequent endothelial dysfunction. From these findings, one might speculate that the exercise training–induced downregulation of NAD(P)H oxidase expression and activity and ROS production leads to improved endothelial function. This hypothesis is supported by the negative linear correlation between endothelial function measured *in vivo* and the biochemical measurements of NAD(P)H activity or ROS formation documented in the present study.

### Clinical Implications

Vascular oxidative stress is increasingly recognized as a key factor for the initiation and acceleration of atherogenesis. On the functional level, elevated ROS concentrations increase premature breakdown of endothelium-derived NO to ONOO<sup>-</sup>, resulting in endothelial dysfunction. On the molecular level, activation of redox-sensitive genes leads to higher expression of adhesions factors like ICAM-1, VCAM-1, and MCP-1, which induce and promote local inflammatory activation and cell adhesion, a prerequisite for plaque formation.<sup>34</sup>

By reducing NAD(P)H oxidase–mediated ROS formation, exercise training interrupts this preclinical process of atherogenesis. In addition, it mimics the effects of AT<sub>1</sub> blockers by reducing AT<sub>1</sub>-R expression and the AT<sub>1</sub>/AT<sub>2</sub>-R ratio. AT<sub>1</sub>-R is involved in multiple steps of atherosclerosis, including monocyte adhesion and activation, endothelial cell apoptosis, vascular smooth muscle proliferation, LDL oxidation, and thrombogenic effects. By interfering with the AT<sub>1</sub>-mediated cascade of atherosclerosis, exercise training recruits an additional vasoprotective mechanism.

### Study Limitations

Despite the observation that exercise training alters the AT<sub>1</sub>-to-AT<sub>2</sub> ratio and the possibility that blood pressure may be influenced by the expression level of AT<sub>1</sub> and AT<sub>2</sub>,<sup>35</sup> the

possibility remains that the changes in ROS and NAD(P)H oxidase observed in the present study result from changes in blood pressure in the training group. However, in vitro cell culture models in which changes in hydrostatic pressure are eliminated indicate that addition of Ang II leads to rapid induction of ROS production, which can be inhibited by AT<sub>1</sub> antagonists or NAD(P)H oxidase inhibitors.<sup>5,13</sup>

In the present study, as in all our previous trials with a similar design,<sup>14,15</sup> hospitalization of only the training group was not associated with any changes in dietary habits, cardiovascular risk profiles, and medications. Nevertheless, we cannot completely rule out the possibility that hospitalizing the training group has partially influenced the results independently of the training effects.

In conclusion, the results of this study provide evidence that exercise training has a positive influence on the antioxidative capacity via the downregulation of AT<sub>1</sub>-R and subsequently by reduced expression and activity of NAD(P)H oxidase. This lower NAD(P)H oxidase enzyme activity and generation of ROS resulted in attenuated Ang II-induced vasoconstriction.

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