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Tatsiana Suvorava, Nadine Lauer, and Georg Kojda

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# Physical Inactivity Causes Endothelial Dysfunction in Healthy Young Mice

Tatsiana Suvorava, PHD, Nadine Lauer, PHARM.D, PHD, Georg Kojda, PHARM.D, PHD

Duesseldorf, Germany

<b>OBJECTIVES</b>	We sought to determine if physical inactivity affects endothelial function in young healthy individuals.
<b>BACKGROUND</b>	Recent studies have linked exercise training to increased bioavailability of vascular nitric oxide (NO) and to improved endothelial function in patients with cardiovascular disorders. The effects of physical inactivity on normal vascular endothelial function are not known.
<b>METHODS</b>	Healthy young male C57Bl/6 mice living in groups of five in large cages, where they were running, climbing, and fighting during their active cycle, were randomly assigned to stay there or to live alone in small cages where they were predominantly resting. After five and nine weeks citrate synthase activity (a measure of mitochondrial respiratory chain activity), heart weight/body weight ratio, vascular reactivity, and protein expression of endothelial nitric oxide synthase (eNOS) were assessed.
<b>RESULTS</b>	Singularized mice showed a reduction of citrate synthase activity ( $p < 0.05$ ), of endothelium-dependent vasorelaxation (to $65 \pm 5\%$ of control levels; $p < 0.001$ ), and of eNOS protein expression (to $53 \pm 8\%$ of control levels; $p < 0.01$ ). In striking contrast, vascular responses to potassium chloride, phenylephrine, and the NO-donor racemic S-nitroso-N-acetylpenicillamine were unchanged. The alterations of vascular eNOS-activity were completely reversible when singularized mice underwent exercise. In mice living in groups, exercise showed only a small effect on aortic eNOS expression.
<b>CONCLUSIONS</b>	In young healthy individuals physical inactivity induces endothelial dysfunction, which is completely reversible by a short period of moderate exercise training. We suggest that physical inactivity, the so-called sedentary lifestyle, increases cardiovascular risk in young healthy individuals by inducing endothelial dysfunction. (J Am Coll Cardiol 2004;44:1320-7) © 2004 by the American College of Cardiology Foundation

Physical inactivity, the so-called sedentary lifestyle, increases cardiovascular morbidity and mortality, as indicated by studies comparing individuals with different levels of daily exercise (1,2). Regarding the underlying molecular mechanism, exercise training is associated with significant physiologic adaptations involving skeletal muscle, cardiac muscle, circulating blood volume, and a variety of metabolic modifications (3). Recently, it has become apparent that exercise induces an increased expression of vascular endothelial nitric oxide synthase (eNOS) as well (4,5). This adaptation most likely improves the bioavailability of endogenous nitric oxide (NO) in mice and humans (5-7). At the same time, exercise induces the expression of extracellular superoxide dismutase (8), which may contribute to the improvement of NO bioavailability.

Bioavailability of endogenous NO may determine exercise capacity by its vasodilatory action. In addition, it has been established that NO not only produces vasodilation but also inhibits platelet aggregation and has antioxidant, antiproliferative, and antiapoptotic properties (9). These effects suggest that increased NO production induced by exercise may also slow the progression of vascular disease

(3). Pharmacologic inhibition of eNOS or eNOS gene disruption has been shown to accelerate the atherosclerotic process (10,11), whereas treatment with NO donors such as organic nitrates can reduce lesion formation, vascular superoxide production, oxidation of low-density lipoprotein, and endothelial dysfunction in cholesterol-fed rabbits (12,13).

It is unknown if physical inactivity affects endothelial function in healthy young individuals. Most epidemiologic and prospective clinical trials investigated the effect of exercise on disease progression in patients with coronary artery disease and heart failure, where endothelial function is already impaired (14). In contrast, the Multiple Risk Factor Intervention Trial (MRFIT) study surveyed 12,138 men without cardiovascular disease for 16 years and found a significantly reduced incidence of cardiovascular deaths in men with normal daily physical activity compared to men with a sedentary lifestyle (1). We speculated that an impairment of endothelial function may be a factor associated with a sedentary lifestyle in healthy individuals and investigated if physical inactivity affects endothelial function in young normal mice.

## METHODS

**Moving and sedentary mice.** A total of 81 male C57Bl/6 mice, four to six months of age, were used for the main study. The study consisted of three arms: 1) a pilot study to assess potential differences in training effects on heart weight/body weight ratio; 2) an exercise restriction arm to

From the Institut fuer Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universitaet, Duesseldorf, Germany. This study was supported by the Forschungskommission der Heinrich-Heine-Universitaet Duesseldorf (Project 9772 109). The first two authors contributed equally to this work.

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#### Abbreviations and Acronyms

ACh	= acetylcholine
ANOVA	= analysis of variance
eNOS	= endothelial nitric oxide synthase
KCl	= potassium chloride
NO	= nitric oxide
pD <sub>2</sub>	= half maximal effective concentration in $-\log$ mol/l
PE	= phenylephrine
SNAP	= racemic S-nitroso-N-acetylpenicillamine

assess the effects of a sedentary lifestyle on endothelial function and citrate synthase activity; and 3) a training intervention arm to assess the reversibility of changes induced by activity restriction. A total of 19 mice were used in preliminary studies to determine changes of heart weight/body weight ratio. Fourteen of these mice were singularized for five weeks and seven of them underwent three weeks of exercise. All mice were bred at the university's animal facilities in a specified pathogen-free area. After weaning, mice from different litters were housed together in groups of five males in cages with a floor space of 900 cm<sup>2</sup> (moving mice) (Fig. 1) at a temperature of 18°C to 20°C, a humidity of 50% to 60%, and a day-night rhythm of 12 h. Mice received food and water (pH 3) ad libitum. Fifty-two of 81 mice were randomly assigned to live alone in small cages (sedentary mice) with a floor space of 300 cm<sup>2</sup> (Fig. 1) for five weeks (n = 31) and nine weeks (n = 21). Some mice of the five-week singularized group (n = 10) and the nine-week singularized group (n = 7) and of the moving group (n = 5) underwent a controlled eight to 15 days' lasting exercise program. All other conditions were identical. Mice living in groups were fighting, running, and climbing for the most part of their active daily cycle, whereas singularized mice were predominantly resting during their active daily cycle and showed a low physical activity, as evidenced by measuring the heart weight/body weight ratio and skeletal and cardiac citrate synthase activity.

Permission for this study was provided by the regional



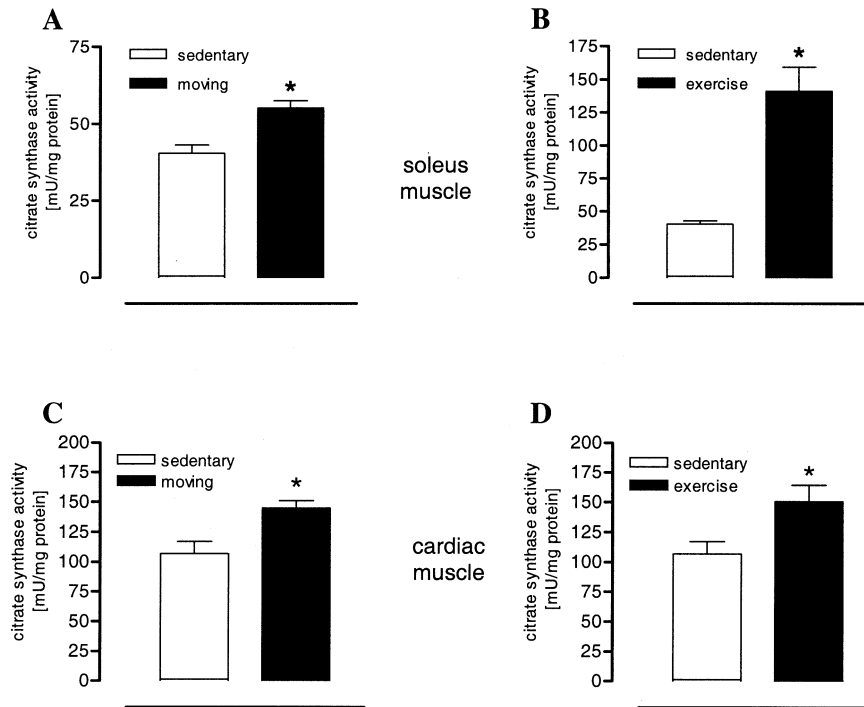
**Figure 1.** Details of the cages used to house mice in groups (bottom cage) and singularized mice (top cage).

government and the experiments were performed according to the guidelines for the use of experimental animals as given by “Deutsches Tierschutzgesetz” and the “Guide for the Care and Use of Laboratory Animals” of the U.S. National Institutes of Health.

**Exercise protocol.** Mice were exercised following a previously established protocol (5). Briefly, mice ran in a newly established self-build exercise treadmill especially designed for mice. Five animals were studied simultaneously. Mice were initially trained three times for 10 min every other day for 10 days. The maximal velocity of the treadmill was 0.25 m/s. After this training, mice were exercised for three weeks at five days a week for 30 min at 0.25 m/s. Thus, eight days of training corresponded to a total time of 10 days (2 days without training) and 15 days of training to a total time of three weeks. The training was executed during the active cycle of the animals. Non-exercised controls were exposed to the same noise and the vibration of the environment. All mice completed the exercise protocol without signs of exhaustion. There was no obvious difference in exercise performance between sedentary and moving mice. Within 16 to 20 h after termination of the last training, mice were sacrificed by inhalation of carbon dioxide and their aortas and hearts were immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting.

**Measurement of citrate synthase activity.** Citrate synthase activity was measured according to Kusnetzov et al. (15). Briefly, soleus muscle and left ventricular cardiac muscle were harvested and snap-frozen in liquid nitrogen. Samples were homogenized in triton X-100 (0.1 % V/V) containing tris buffer (0.1 M, pH 8.1) and then repeatedly thawed and refrozen to further disrupt mitochondria. Five  $\mu$ l of the homogenate was used to start the reaction (generation of the mercaptide) in a volume of 1 ml aqueous reaction volume containing 5,5-dithiobis(2-nitrobenzoate) (DTNB, 0.1 mM), acetyl-CoA (0.3 mM), oxaloacetate (0.5 mM) and triton X-100 (0.25 % V/V). Changes in absorbance at 412 nm were measured at 30°C for 5 min. Total protein levels in the homogenate was determined by the Bradford method (16) and citrate synthase activity is given in mU/mg total protein.

**Vasorelaxation studies.** Preparation of thoracic ring segments was performed in HEPES-containing Krebs-Henseleit buffer, whereas the organ bath experiments were done in the same buffer lacking HEPES. After a 60-min equilibration period, aortic rings were repeatedly subjected to 80 mmol/l potassium chloride (KCl). The vasoconstriction that developed during the last of three KCl applications was taken as the maximal receptor-independent vasoconstriction. Function of endothelium was examined by cumulative addition of acetylcholine (ACh) ( $10^{-9}$  to  $10^{-5}$  mol/l) following submaximal precontraction with phenylephrine (PE). In moving mice and those subjected to five weeks of singularization, endothelium-dependent vasodilation was followed by a cumulative application of PE ( $10^{-9}$ – $10^{-5}$  mol/l). Thereafter, the aortic rings



**Figure 2.** Activity of citrate synthase in homogenates of soleus (A, B) and cardiac left ventricular muscle (C, D) of singularized mice (sedentary, n = 7, all panels), mice living in groups (moving, n = 5) (A, C) and singularized mice that underwent three weeks of endurance training (exercise, n = 7) (B, D) (\*p < 0.05, unpaired two-tailed Student *t* test).

were precontracted with 1- $\mu$ mol/l PE and a concentration-response curve for racemic S-nitroso-N-acetylpenicillamine (SNAP) ( $10^{-9}$ – $10^{-5}$  mol/l) was performed.

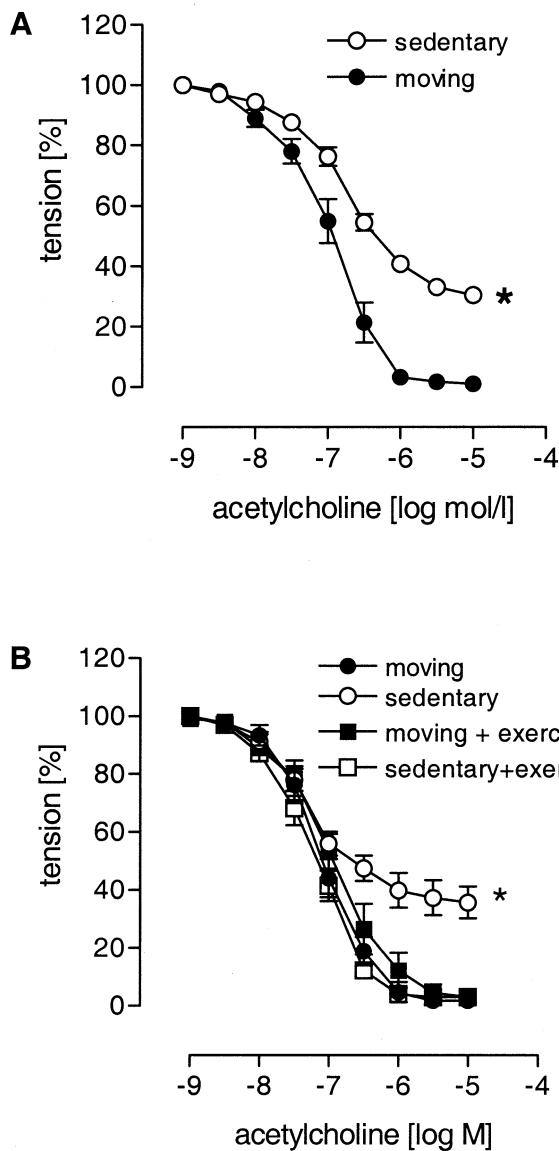
**Determination of aortic eNOS expression.** Western blot analysis was done in a blinded fashion. Frozen aortas from moving, sedentary (five weeks), and exercised mice were immersed in iced tris-buffer (5 mmol/l, pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aprotinin, phenylmethylsulfonyl fluoride, and antipain (10  $\mu$ g/ml). The tissues were homogenized for 30 s using a polytron homogenizer. The homogenates were then centrifuged for 10 min at  $100 \times g$  to remove particulate matter and unbroken cells. Total protein levels were determined by the Bradford method (16). Western blot analysis was performed as described previously (5) using a commercially available monoclonal antibody (Transduction Laboratories, Lexington, Kentucky) and the enhanced chemiluminescence detection system (Amersham).

**Substances and solutions.** Racemic S-nitroso-N-acetylpenicillamine was synthesized in our laboratory as described previously (17). All other chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany) in analytical grade. The stock solutions of ACh (10 mmol/l), PE (10 mmol/l), and KCl (4 mol/l) were prepared in distilled water. Solutions of SNAP (200 mmol/l) were prepared in dimethylsulfoxide. All stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice, and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

**Statistics.** All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0; analysis of variance [ANOVA]) and are expressed as mean values and standard error of the mean. Significant differences were evaluated using either unpaired two-tailed Student *t* test, ordinary two-way-ANOVA (without post-tests) and one-way-ANOVA with subsequent Newman-Keuls multiple comparison test. A p value below 0.05 was considered significant.

## RESULTS

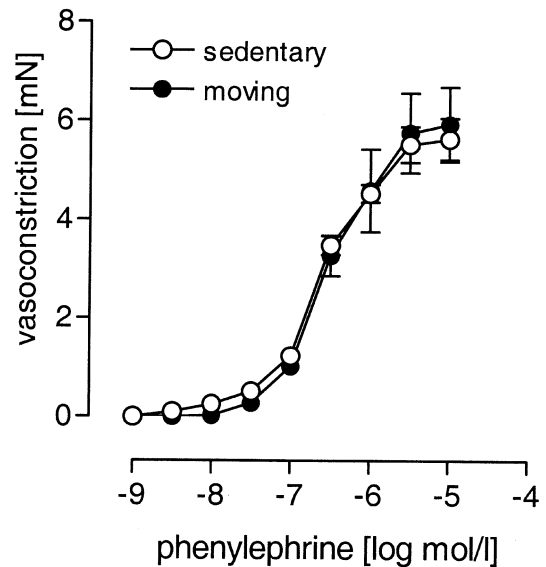
**Quantitation of physical activity.** There was a significant increase of soleus muscle citrate synthase activity in moving and exercised mice as compared with sedentary mice (Figs. 2A and 2B), and this increase was much more pronounced after exercise. Likewise, citrate synthase activity was enhanced in cardiac muscle of moving and exercised mice. A comparison between the two muscle types reveals some differences. As expected, the citrate synthase activity was greater in cardiac muscle of sedentary mice as compared with soleus muscle, suggesting a higher mitochondrial activity in the former tissue. Furthermore, the increase of citrate synthase activity in cardiac muscle was identical in moving and exercised mice (Figs. 2C and 2D), suggesting an already high activity as previously reported (18). To further quantify the level of physical activity, we determined the body weight/heart weight ratio in sedentary mice with (n = 7) and without (n = 7) three weeks of exercise and in moving mice (n = 5). We found that the heart weight/body



**Figure 3.** Endothelium-dependent vasodilation in aortic rings of sedentary mice after (A) five weeks of singularization (n = 6) compared with mice living in groups of five animals per cage (moving mice, n = 6) and (B) after nine weeks of singularization (n = 7) compared with moving mice (n = 5) and with sedentary (n = 7) and moving mice (n = 5) that underwent the exercise program. Physical inactivity induced by singularization resulted in a significant impairment of endothelium-dependent vasorelaxation (\* = p < 0.001, analysis of variance). Three weeks of exercise completely reversed this impairment, whereas exercise training had no effect on endothelium-dependent vasodilation in moving mice.

weight ratio (in mg/g) of sedentary mice ( $5.2 \pm 0.09$ ) was increased in moving mice ( $5.5 \pm 0.1$ ; p < 0.05) and after three weeks of exercise ( $6.04 \pm 0.04$ ; p < 0.01).

**Endothelium-dependent vasodilation.** Endothelial function of moving and sedentary mice was assessed by examination of endothelium-dependent vasodilation to ACh. The concentration-response curves for ACh demonstrate that five (Fig. 3A) and nine weeks (Fig. 3B) of forced physical inactivity as initiated by singularization in small cages results in endothelial dysfunction. After five weeks of singularization we found a reduction of the maximal vasodilation to 10



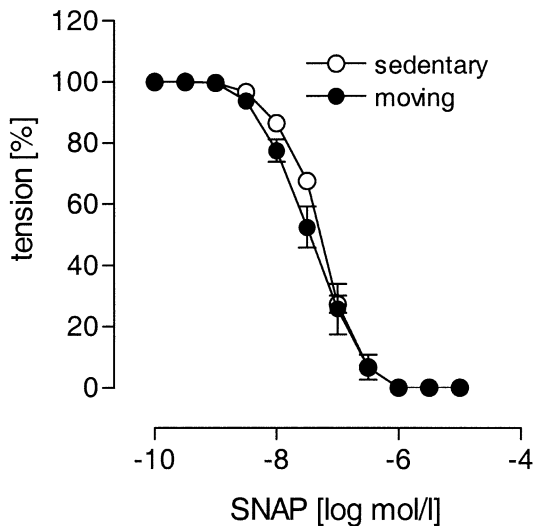
**Figure 4.** Vasoconstrictor response to increasing concentrations of phenylephrine in aortic rings of singularized sedentary mice (n = 6) and moving mice (n = 6). There was no difference between the groups (p = 0.6262, analysis of variance).

$\mu\text{mol/l}$  ACh from  $99.4 \pm 0.3\%$  in moving mice to  $69.4 \pm 1.9\%$  in sedentary mice (p < 0.0001). After nine weeks of singularization this vasodilator response was reduced from  $91.7 \pm 2.7\%$  in moving mice to  $64.3 \pm 5.5\%$  in sedentary mice (p = 0.0008). These data suggest that five weeks of physical inactivity is sufficient to induce a degree of endothelial dysfunction in mice that is not further aggravated by prolonging the time period of physical inactivity. Sedentary mice that underwent the exercise program regained their normal ACh response, whereas exercise had no effect on endothelium-dependent vasorelaxation in moving mice (Fig. 3B).

**Vasocontractile responses.** Vasocontractile responses were not different in moving and sedentary mice. Subjection of aortic rings to a single dose of 80 mmol/l KCl resulted in an identical rise of maximal vascular tension in sedentary ( $6.4 \pm 0.4$ , n = 6) and moving mice ( $6.6 \pm 0.4$ , n = 6, p = 0.728). Likewise, neither the half maximal effective concentration in  $-\log \text{mol/l}$  ( $\text{pD}_2$ ) values for PE in moving ( $6.479 \pm 0.21$ ) and sedentary mice ( $6.569 \pm 0.12$ , p = 0.7157) nor the maximally inducible vasoconstrictions different (p = 0.7919) (Fig. 4).

**NO-dependent vasodilation.** Contrary to endothelium-dependent vasodilation, the NO-dependent vasodilation induced by the NO donor SNAP showed identical  $\text{pD}_2$  values in moving ( $7.393 \pm 0.071$ ) and sedentary mice ( $7.302 \pm 0.03422$ , p = 0.2180) (Fig. 5). These data suggest that an impairment of the NO/cGMP-pathway is most likely not involved in endothelial dysfunction induced by a sedentary lifestyle in mice.

**Aortic eNOS protein expression.** Assessment of eNOS protein expression showed a significant reduction in sedentary mice that was evident in each animal examined (relative



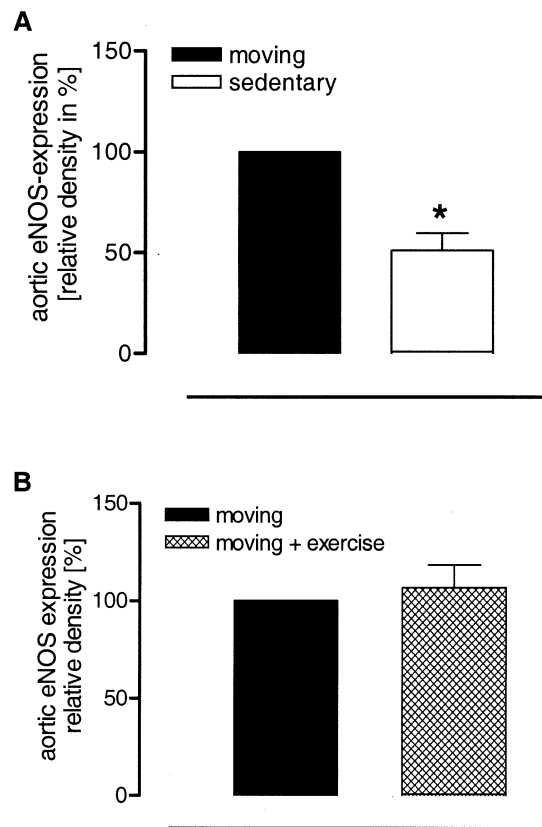
**Figure 5.** Vasodilator response to increasing concentrations of the nitric oxide donor racemic S-nitroso-N-acetylpenicillamine in aortic rings of singularized sedentary mice (n = 6) and of moving mice (n = 6). There was no difference between the groups (p = 0.4059, analysis of variance).

densities ranging from 0.31 to 0.64; p < 0.01) (Fig. 6A). These data suggest that the overall halved expression of eNOS contributes to the impairment of endothelium-dependent vasodilation induced by physical inactivity.

**Effect of exercise on eNOS expression in sedentary and moving mice.** Sedentary mice were exercised by a standard protocol for eight days and 15 days. Overall, there was a time-dependent several-fold increase of aortic eNOS-protein expression (p = 0.0147, ANOVA) (Fig. 7A). Subsequent Newman-Keuls multiple comparison tests of the data depicted in Figure 7A revealed a significant difference only for the comparison sedentary versus 15 days of exercise. Although a similar trend was observed in left ventricular myocardium, the increase of eNOS protein expression was not significant in this tissue (p = 0.1637, ANOVA) (Fig. 7B). In striking contrast, aortic eNOS protein expression did not change after 15 days of exercise in moving mice (p > 0.05) (Fig. 6B).

## DISCUSSION

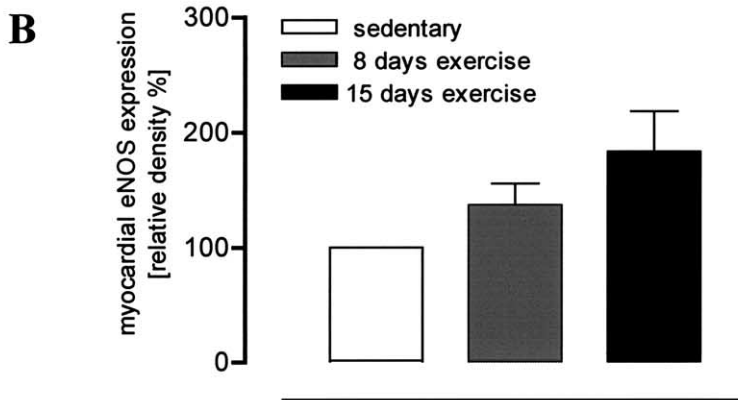
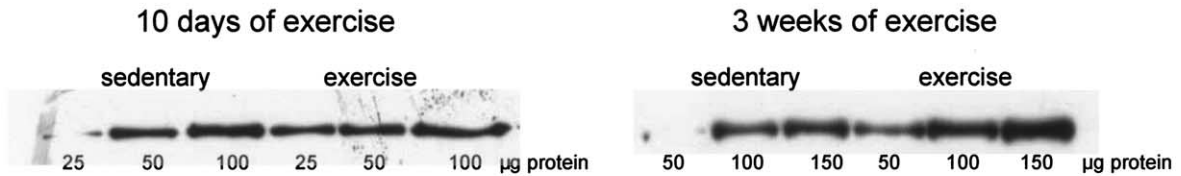
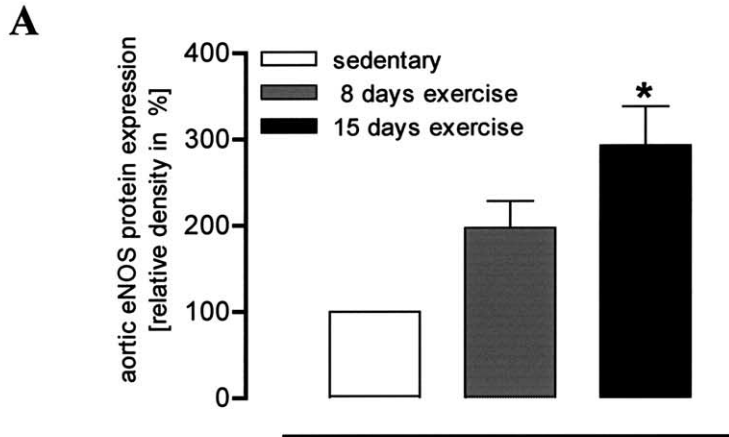
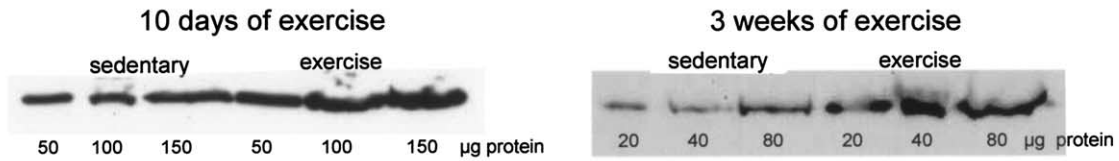
The aim of this study was to determine the influence of physical inactivity on vascular endothelial function. We demonstrate for the first time that a sedentary lifestyle, mimicked by forced physical inactivity in young healthy mice, can induce a specific impairment of endothelium-dependent vasodilation, whereas vasocontractile activity and NO-donor-induced vasorelaxation were not changed. Further experiments showed that a reduction of vascular eNOS protein expression most likely contributes to this endothelial dysfunction. When sedentary mice underwent exercise, the expression of eNOS increased by severalfold, whereas exercise had only a small effect on aortic eNOS-expression in moving mice. Thus, our data suggest that physical inactivity can reduce vascular expres-



**Figure 6.** (A) Protein expression of eNOS in aortic rings of singularized sedentary mice (n = 5) compared with moving mice (n = 5) and (B) of moving mice (n = 4) compared with moving mice that underwent an exercise program (n = 4). Singularization resulted in a significant reduction of eNOS protein expression (A, p < 0.01), whereas exercise had no effect on eNOS protein expression in moving mice (B, p > 0.05, unpaired two-tailed Student t test).

sion of eNOS and thereby elicit endothelial dysfunction in the absence of other cardiovascular risk factors.

Endothelial dysfunction is a well-known pathologic condition that is associated with a variety of cardiovascular diseases such as coronary artery disease, hypertension, heart failure, and diabetes. The mechanism of endothelial dysfunction is multifactorial and most likely depends on the underlying pathologic process. It is generally accepted that vascular inflammation, vascular oxidative stress, and aging are important factors in cardiovascular diseases (19-21). Here we describe for the first time the development of endothelial dysfunction in healthy young mice with no signs of vascular inflammation or oxidative stress that had been subjected to forced physical inactivity. At the same time, the protein expression of endothelial nitric oxide synthase was reduced by one-half, whereas activation of the NO/cGMP pathway by an NO donor was equally effective in sedentary and moving mice. Therefore, downregulation of eNOS expression appears to be a key mechanism underlying the impairment of endothelial function in young healthy sedentary mice. However, in cardiovascular patients other mechanisms such as increased eNOS phosphorylation have been shown to follow vigorous exercise (7). Other mecha-



**Figure 7.** Effect of 8 days ( $n = 5$ ) and 15 days of exercise ( $n = 5$ ) on eNOS-protein expression in (A) aortic rings and (B) left ventricular myocardium of singularized sedentary mice ( $n = 5$  for each exercise period). Although exercise induced a significant and time-dependent upregulation of eNOS protein expression in aortic rings ( $p = 0.0147$ , analysis of variance), there was only a trend in left ventricular myocardium ( $p = 0.1637$ ). Subsequent Newman-Keuls multiple comparison test of the data depicted in A revealed a significant difference only for the comparison sedentary versus 15 days' exercise as indicated (\*,  $p < 0.05$ , analysis of variance).

nisms decreasing the bioavailability of endothelial NO in sedentary mice, such as increased oxidation by superoxide radicals owing to a reduced expression of extracellular superoxide dismutase, might also play a role (8).

Many different conditions, endogenous mediators, and drugs have been shown to modulate the expression of eNOS (22). Among these, mechanisms modifying eNOS expression in response to exercise might be particularly important, because it seems conceivable that a reversion of these mechanisms occurs in sedentary mice. In accordance, we demonstrate that exercise reversed endothelial dysfunction induced by physical inactivity. Recent reports suggest that there are at least two different mechanisms underlying the upregulation of eNOS expression by exercise. First, exercise increases heart rate, which in turn increases blood flow and most likely vascular shear stress. Besides its role as the most important physiologic activator of endothelial NO production, shear stress has been shown to increase vascular eNOS expression (23,24). Further studies suggested that this process is dependent on c-Src and involves an increase of transcription and of messenger ribonucleic acid stability as well (25). Thus, a decreased intensity of physiologic shear stress, as expected in sedentary mice, might diminish vascular eNOS expression by reducing the shear stress-dependent activity of c-Src in endothelial cells.

Second, exercise increases not only oxygen consumption but also the generation of reactive oxygen species such as superoxide and hydrogen peroxide (26). It is well known that superoxide generation is increased in a nonenzymatic fashion during adenosine triphosphate synthesis by an electron transfer from coenzyme Q to molecular oxygen (27). Furthermore, and likely more important, shear stress has been shown to increase the vascular generation of reactive oxygen species by an endothelium-dependent mechanism (28). A later analysis of this phenomenon showed an activation of endothelial nicotinamide adenine dinucleotide phosphate, reduced form oxidase as a possible underlying cause (29). Although superoxide barely traverses cell membranes and is rapidly converted by superoxide dismutases, the resulting product hydrogen peroxide can diffuse through the vascular wall and is much more stable (20). Previous data have shown that hydrogen peroxide can increase the expression and activity of eNOS by phosphorylation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II/janus kinase 2 (30,31). By generating transgenic mice with an endothelial-specific overexpression of catalase, we have recently provided evidence that hydrogen peroxide contributes to exercise-induced upregulation of eNOS. In these transgenic mice, three weeks of exercise performed according to the protocol used in the present study had no effect on vascular eNOS expression (32).

Western blotting for eNOS was done in a blinded fashion, but the organ bath studies were not. This might be considered a limitation of our study. However, in striking contrast to the observed endothelial dysfunction, the vascular responses to the vasoconstrictors KCl and PE and to the vasodilatory activity of

the NO donor SNAP were normal. These data demonstrate that five weeks of forced physical inactivity has no impact on receptor-independent and alpha-adrenergic vasoconstriction. In addition, there seems to be no functionally important impairment of the vascular NO/cGMP pathway. In contrast, vascular oxidative stress and inflammatory stimuli such as cytokines, which typically induce endothelial dysfunction, are known to increase PE responses, presumably by activation of protein kinase C (33,34). Furthermore, oxidative stress and atherosclerosis have been shown to strongly impair the NO/cGMP pathway by inhibition of the catalytic activity of soluble guanylyl cyclase (35). In view of these studies, it is unlikely that vascular inflammation and/or oxidative stress have caused endothelial dysfunction induced by forced physical inactivity in healthy young mice.

Regarding the wildlife habits, singularization of mice is an artificial situation. This holds also true for mice being caged in groups. Interestingly, we found identical endothelium-dependent vasodilation in exercised and moving mice, although there was a large and significant difference concerning citrate synthase activity, which is known to correlate with physical activity (18). This suggests that low-intensity physical activity may be sufficient to maintain normal endothelial function in young healthy individuals. Furthermore, vigorous exercise has no further effect on endothelium-dependent vasodilation and eNOS expression in normally active mice. Thus, it seems likely that the differences in physical activity in our experimental setup mimic to a certain extent the situation in humans. For example, it was shown that there is an identical rate of cardiovascular events between women who simply walked for exercise and those who underwent vigorous exercise (2).

Recent data suggest that endothelial dysfunction is an independent predictor of cardiovascular event rates (36-38). Thus, endothelial dysfunction induced by a sedentary lifestyle might be an important pathophysiologic event. Our data suggest that this unfavorable change of vascular function can be prevented or remarkably delayed in young individuals by either a daily short-lasting high-intensity training period or continuous low-intensity physical activity. Based on our observations, we propose that regular physical activity may exert beneficial effects in two different ways. In patients with cardiovascular disease, exercise reduces the degree of endothelial dysfunction, whereas in young healthy individuals normal physical activity and/or moderate exercise might delay the development of cardiovascular disorders by maintaining normal endothelial function.

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**Reprint requests and correspondence:** Dr. Georg Kojda, Institut fuer Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universitaet, Moorenstr. 5, 40225 Duesseldorf, Germany. E-mail: kojda@uni-duesseldorf.de.

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**Physical inactivity causes endothelial dysfunction in healthy young mice**

Tatsiana Suvorava, Nadine Lauer, and Georg Kojda

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