Physical Inactivity Increases Oxidative Stress, Endothelial Dysfunction, and Atherosclerosis

Ulrich Laufs, Sven Wassmann, Thomas Czech, Thomas Münzel, Marco Eisenhauer, Michael Böhm, Georg Nickenig

Objective—Sedentary lifestyle is associated with increased cardiovascular events. The underlying molecular mechanisms are incompletely understood. Reactive oxygen species (ROS) contribute to endothelial dysfunction and atherosclerosis. An important source of vascular ROS is the NADPH oxidase.

Methods and Results—C57BL6 mice were subjected to regular housing (physical inactivity) or voluntary training on running wheels (6 weeks). Inactivity increased vascular lipid peroxidation to 148±9% and upregulated superoxide release to 176±17% (L-012 chemiluminescence) and 188±29% (cytochrome C reduction assay), respectively. ROS production was predominantly increased in the endothelium and the media (dihydroethidium fluorescence). Activity of the NADPH oxidase was increased to 154±22% in the sedentary group. Rac1 GST-PAK pull-down assays showed an upregulation of rac1 activity to 161±14%. Expression levels of the subunits nox1, p47phox, and p67phox were increased. To address the significance of the antioxidative effects of running, experiments were repeated in apolipoprotein E–deficient mice treated with a high-cholesterol diet. Inactivity increased vascular superoxide production and impaired endothelium-dependent vasorelaxation. Atherosclerotic lesion formation was significantly accelerated in sedentary mice.

Conclusions—Inactivity increases vascular NADPH oxidase expression and activity and enhances vascular ROS production, which contributes to endothelial dysfunction and atherosclerosis during sedentary as opposed to physically active lifestyle. (Arterioscler Thromb Vasc Biol. 2005;25:809-814.)

Key Words: physical inactivity ■ exercise ■ oxidative stress ■ endothelial dysfunction ■ atherosclerosis

The prevalence of physical inactivity in adults is increasing. Nearly 40% of the adult population subject themselves to <10 minutes of continuous physical activity per week. These lifestyle changes are of special interest because physical inactivity has been proposed as an independent cardiovascular risk factor. However, the molecular mechanisms leading from sedentary lifestyle to cardiovascular events are poorly defined.

On the other hand, prospective epidemiological data indicate that moderate (eg, walking) and vigorous exercise are associated with substantial reductions in the incidence of cardiovascular events. Physical training improves endothelial function, exercise capacity, and collateralization in patients with coronary artery disease and prevents the progression of carotid atherosclerosis. Among other beneficial effects, physical activity is associated with improved mood, body weight, blood pressure, insulin sensitivity, and hemostatic and inflammatory variables.

Reactive oxygen species (ROS) play a pivotal role in the pathogenesis of endothelial dysfunction and atherosclerosis. Acutely, intensive exercise has been shown to increase oxidative stress because increased aerobic metabolism is a source of oxidative stress, whereas long-term moderate exercise may upregulate antioxidative enzymes and decrease indices of oxidative stress.

The aim of this study was to compare the effects of physical inactivity with voluntary running. Mice were subjected to regular housing or cages equipped with running wheels. We postulated that giving the animals the opportunity of voluntary running resembles their natural habitat more closely and tested the effects of voluntary running compared with sedentary behavior on parameters of vascular oxidative stress. In addition, we used the model of cholesterol-fed apolipoprotein E–deficient (apoE−/−) mice to examine the effects of inactivity versus running on endothelial function and atherosclerotic lesion formation.

Methods

The Methods section is available in the online data supplement, available at http://atvb.ahajournals.org.
The mean running distance of active mice was 4900 ± 700 m per 24 hours. Body weight did not differ between active and inactive mice (24 ± 0.3 versus 23 ± 0.3 g). As a global parameter of oxidative stress, lipid peroxidation of the aortic wall was compared. Inactive mice displayed upregulation of vascular lipid hydroperoxides to 148 ± 9% compared with active mice (n = 6 per group; P < 0.05; Figure 1A). Because superoxide radicals are involved in the pathogenesis of atherosclerosis, we determined aortic superoxide production by 2 different methods. As shown in Figure 1B and 1C, vascular superoxide release was significantly increased in inactive compared with active mice (176 ± 17% as assessed by L-012 chemiluminescence [n = 8 per group; P < 0.05] and 182 ± 29% as determined by cytochrome C reduction assays [n = 4 per group; P < 0.05], respectively). Because NADPH oxidase is a major source of superoxide radicals in the vascular wall, we assessed NADPH oxidase activity in aortic tissue. Figure 1D displays that NADPH oxidase activity was upregulated to 154 ± 22% (n = 6 per group; P < 0.05) in the sedentary group.

Upregulation of NADPH Oxidase Subunits in Inactive Mice

Translocation of the cytosolic regulatory subunits p47phox, p67phox, and the small GTP-binding protein rac1 to the plasma membrane is a prerequisite for NADPH oxidase activation and subsequent ROS production. Rac1 GTPase activity in aortic homogenates was determined by rac1 GST-PAK pull-down assays. A and B, Representative example (A) and densitometric quantification (B; mean ± SEM; n = 6 per group; *P < 0.05 vs active mice). Aortic membrane and cytosolic protein expression of the NADPH oxidase subunits p47phox and p67phox and the housekeeping gene β-actin were determined by Western blot analysis (n = 6 per group). D, Representative blots.
To evaluate the localization of increased ROS formation and NADPH oxidase subunit expression within the aortic wall, dihydroethidium (DHE) fluorescence microscopy and immunohistochemical stainings of aortic sections were performed. These experiments revealed that ROS production, as well as protein expression of the NADPH oxidase subunits p47phox and p67phox, was predominantly increased in the endothelium and the media of the aortic wall of inactive mice as opposed to active animals (Figure 1, available online at http://atvb.ahajournals.org).

Physical Inactivity Impairs Endothelium-Dependent Vasorelaxation

To address the significance of the pro-oxidative effects of physical inactivity for vascular pathology, experiments were repeated with apoE<sup>−/−</sup> mice treated with a high-cholesterol diet and were in parallel subjected to voluntary exercising (active) or sedentary lifestyle (inactive). Aortic segments of these apoE<sup>−/−</sup> mice and of wild-type mice were isolated and their functional performance was assessed in organ chamber experiments. A and B, Endothelium-dependent vasorelaxation induced by carbachol (A) and endothelium-independent vasodilation induced by nitroglycerin (B) expressed in percentage of maximal phenylephrine-induced vasoconstriction (mean±SEM; n=6 to 8 per group; *P<0.05 vs inactive apoE<sup>−/−</sup> mice; #P<0.05 vs active and inactive apoE<sup>−/−</sup> mice).

Figure 3. Endothelium-dependent and -independent vasorelaxation. ApoE<sup>−/−</sup> mice were treated with a high-cholesterol diet and were in parallel subjected to voluntary exercising (active) or sedentary lifestyle (inactive). Aortic segments of these apoE<sup>−/−</sup> mice and of wild-type mice were isolated and their functional performance was assessed in organ chamber experiments. A and B, Endothelium-dependent vasorelaxation induced by carbachol (A) and endothelium-independent vasodilation induced by nitroglycerin (B) expressed in percentage of maximal phenylephrine-induced vasoconstriction (mean±SEM; n=6 to 8 per group; *P<0.05 vs inactive apoE<sup>−/−</sup> mice; #P<0.05 vs active and inactive apoE<sup>−/−</sup> mice).

To address the significance of the pro-oxidative effects of physical inactivity for vascular pathology, experiments were repeated with apoE<sup>−/−</sup> mice treated with a high-cholesterol diet. Figure 3 shows that inactive apoE<sup>−/−</sup> mice developed severe endothelial dysfunction compared with wild-type animals (n=6 per group; P<0.05), as demonstrated by impaired endothelium-dependent vasorelaxation in isolated aortic segments, whereas endothelium-independent vasodilation was not altered. In contrast, voluntary running significantly improved endothelium-dependent vasorelaxation (n=8 per group; P<0.05).

Atherosclerotic Plaque Formation Is Accelerated in Sedentary ApoE<sup>−/−</sup> Mice

ApoE<sup>−/−</sup> mice developed atherosclerotic lesions in the aortic root and ascending aorta after 6 weeks of treatment with high-cholesterol diet. A representative example of an inactive and an active apoE<sup>−/−</sup> mouse is shown in Figure 4A. Histomorphometric analysis revealed that physically inactive apoE<sup>−/−</sup> mice experienced significantly accelerated atherosclerotic lesion formation in the aortic root and the ascending aorta compared with physically active mice (n=8 per group; P<0.05; Figure 4B).

Physical Inactivity Increases Vascular Oxidative Stress in ApoE<sup>−/−</sup> Mice

Compared with wild-type animals, cholesterol-fed apoE<sup>−/−</sup> mice displayed 2-fold higher vascular superoxide levels, as
assessed by L-012 chemiluminescence (n=8 per group; P<0.05; Figure 4C). Importantly, physical inactivity increased vascular superoxide production even further in the apoE<sup>−/−</sup> mice (188±14% compared with active apoE<sup>−/−</sup> mice; n=8 per group; P<0.05). Similarly, apoE<sup>−/−</sup> animals showed upregulation of vascular NADPH oxidase activity compared with wild-type mice, which was further increased by physical inactivity (n=8 per group; P<0.05; Figure 4D).

Physical Inactivity and Regulation of Endothelial NO Synthase

Voluntary running was associated with marked upregulation of vascular endothelial NO synthase (NOS) mRNA and protein expression and NOS activity in wild-type mice compared with inactive wild-type animals (n=4 to 8 per group; P<0.05; Figure 5A through 5C). Inactive apoE<sup>−/−</sup> mice showed a trend toward lower aortic NOS activity compared with inactive wild-type animals and active apoE<sup>−/−</sup> mice, which was not statistically significant (n=6 per group). In contrast to wild-type mice, voluntary running did not significantly increase NOS activity in apoE<sup>−/−</sup> animals (n=6 per group; Figure 5C). Treatment with N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) inhibited NOS activity in active wild-type- and apoE<sup>−/−</sup> mice (n=6 per group; P<0.05).

Discussion

This study compared oxidative stress, endothelial function, and atherosclerosis between mice kept under usual conditions in regular cages with mice in identical cages supplied with a running wheel. The mice spent considerable time, mostly at night, running in and on the wheels, as reflected by the mean distance of 4.9 km per 24 hours. In contrast to previous studies, neither of the 2 groups was subjected to stress, force, or manipulation of any kind. A second major difference to previously reported experimental training protocols of limited (eg, 30 minutes per day, 5 days per week) intensive exercise (eg, swimming or motorized treadmill) is the moderate but repetitive pattern of exercise chosen voluntarily by the mice. Therefore, we propose that having the opportunity to exercise resembles the natural habitat of mice more closely than cages without running wheels. Consequently, the inactivity of regular laboratory mice has to be considered the experimental intervention in this study. This notion is supported by previous findings that survival rates decrease in sedentary as opposed to exercising rodents.

Five independent assays were applied to assess the effect of physical inactivity on vascular oxidative stress. In wild-type mice, physical inactivity significantly increased vascular lipid peroxidation as a global marker of oxidative stress. More specific analysis of intact aortic rings showed increased superoxide production in sedentary mice, as assessed by L-012 chemiluminescence, superoxide dismutase–inhibitable cytochrome C reduction assays, and DHE staining. This finding may be important because superoxide has been shown to promote endothelial dysfunction and atherosclerosis. Activity of vascular NADPH oxidase was measured because this enzyme represents a major source of superoxide in the vascular wall and was found to be increased in sedentary mice. NADPH oxidase is a multicomponent enzyme complex that consists of the membrane-bound cytochrome b558, which is a heterodimer of gp91phox and p22phox in endothelial cells and nox1 and p22phox in smooth muscle cells, and the cytosolic regulatory subunits p47phox, p22phox, and rac1 GTPase. Translocation of these cytosolic regulatory subunits to the plasma membrane is a prerequisite for oxidase activation and ROS production. Rac1 GTPase has been shown to be a central regulator of NADPH oxidase–induced superoxide release in the vasculature and in the myocardium. In addition, it has been demonstrated that increased expression levels of p22phox and nox1 are associated with enhanced NADPH oxidase activity. Physically inactive mice displayed increased rac1 GTPase activity and enhanced mRNA and membrane protein expression of p47phox and p22phox compared with active mice. Furthermore, expression of nox1 was found to be increased in sedentary mice, whereas expression levels of the subunits p22phox, gp91phox, and nox4 were not different between the groups.

Regulation of rac1 GTPase by physical activity may have implications for the cardiovascular system beyond regulation of NADPH oxidase; however, further studies are needed to address this point.

The increase of superoxide production was not limited to sedentary wild-type animals but was also observed in apoE<sup>−/−</sup> mice despite higher baseline levels of superoxide release. Vascular superoxide production and NADPH oxidase activity were significantly higher in apoE<sup>−/−</sup> mice than in wild-type animals, indicating the potential importance of oxidative stress in the pathology of atherogenesis in this animal model.
In agreement with previous studies, running wild-type mice showed increased endothelial NOS (eNOS) expression. Although eNOS may uncouple under certain conditions and become a superoxide-producing enzyme, the reduction of superoxide production measured in the same samples suggests that NO production and the superoxide-scavenging properties of NO predominate under the exercising conditions of the present study. Meilhac et al found that intensive running on a motorized treadmill 5 days per week for 6 to 12 weeks increased lipid peroxidation and eNOS expression in cholesterol-fed low-density lipoprotein receptor–deficient mice. It is interesting to speculate that this form of intensive short-term exercise may result in uncoupling of eNOS and a pro-oxidant status, whereas a continuous pattern of running does not. This is supported by a recent study showing augmentation of endothelial-dependent vasodilation by moderate-intensity aerobic exercise through increased production of NO but increased oxidative stress after high-intensity exercise. In rats, administration of the eNOS inhibitor l-NAME reduced the benefits of physical training on the vessel wall after balloon injury. In eNOS−/− mice, moderate exercise was shown to worsen energy metabolism in oxidative skeletal muscle. In contrast to wild-type mice, physical exercise did not increase vascular NO activity in apoE−/− mice. It is not clear whether the lack of eNOS responsiveness to physical activity contributes to atherogenesis in this model. The role of eNOS during atherogenesis in apoE−/− mice may be double-edged because overexpression and inhibition have been reported to accelerate lesion formation in apoE−/− mice. These data, together with our findings, show that eNOS-dependent as well as eNOS-independent mechanisms (such as regulation of NADPH oxidase) contribute to the regulation of vascular free radical load and NO bioavailability by physical activity in wild-type and apoE−/− mice. Clearly, additional studies are needed to further dissect the role of eNOS in the pathogenesis of atherosclerosis in apoE−/− mice.

The major finding of this study is the impairment of endothelial function and acceleration of atherosclerosis in inactive animals compared with mice equipped with running wheels. These results suggest that sedentary lifestyle is associated with enhanced vascular oxidative stress, which, in turn, propagates vascular dysfunction. It may be speculated that these mechanisms may contribute to the elevated cardiovascular event rates associated with physical inactivity in humans. In addition, our findings may have implication for the future design of animal studies because the animal husbandry in cages without the possibility to exercise may not reflect the desired baseline condition frequently equated with the control setting. According to the presented data, physical inactivity is a risk factor for vascular disease by promoting NADPH oxidase activity, resulting in increased vascular superoxide release and ultimately vascular dysfunction and atherosclerotic lesion formation. Physical activity is a powerful intervention to improve endothelial function and to prevent progression of atherosclerosis.

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References


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Online Figure, Supplement II. Localization of aortic superoxide formation and NADPH oxidase expression.

In situ-detection of superoxide production was performed by DHE fluorescence microscopy in aortic sections of active and inactive wildtype animals. Immunohistochemical stainings of von Willebrand factor (vWF), nuclei (DAPI), the NADPH oxidase subunits p47phox and p67phox, and isotype controls (control) of corresponding sections. Representative microscopic scans (n=6 per group).
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**Methods - Online Supplement I**

**Animals and exercising**

Animal experiments were conducted in accordance with institutional guidelines and the German animal protection law. Male C57/Bl6 mice (wildtype; Charles River, Sulzfeld, Germany) and male ApoE^{-/-} mice (C57/Bl6 genetic background; Charles River), were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. Mice were randomized to either a sedentary or exercising group. Each exercising mouse was kept in an individual cage supplied with a running wheel (12.8 cm diameter) equipped with a tachometer (Sigma Sport BC401) in order to record the daily running distance. Mice ran voluntarily. The mean running distance did not differ between wildtype and ApoE^{-/-} mice. ApoE^{-/-} mice were randomized to cages with or without running wheels at an age of 12 weeks. At the same time, all ApoE^{-/-} mice were treated with a high-fat, cholesterol-rich diet containing 21% fat, 19.5% casein, and 1.25% cholesterol (Sniff, Soest, Germany). For indicated mice, N^{G}-nitro-L-arginine methyl ester (L-NAME) (Sigma) was added to the drinking water (daily dose, ~50 mg/kg; concentration in drinking water, 1.5 mg/ml). Mice were sacrificed after 6 weeks by atlantoaxial dislocation and tissue samples were harvested immediately.
**Measurement of lipid peroxidation**

Aortic tissue was homogenized in PBS (pH 7.4) containing butylated hydroxytoluene (4 mmol/l). Lipid hydroperoxides were determined using the Lipid Peroxidation Assay Kit II (Calbiochem) and expressed as µmol/mg protein.

**Measurement of vascular superoxide production**

Superoxide release in intact aortic segments was determined by L-012 chemiluminescence. L-012 (Wako Chemicals) is a luminol derivate with high sensitivity for superoxide radicals which does not exert redox cycling itself \(^1,^2\). Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (pH 7.4; NaCl 99.01 mmol/l, KCl 4.69 mmol/l, CaCl\(_2\) 1.87 mmol/l, MgSO\(_4\) 1.20 mmol/l, NaHEPES 20.0 mmol/l, K\(_2\)HPO\(_4\) 1.03 mmol/l, NaHCO\(_3\) 25.0 mmol/l, D(+)Glucose 11.1 mmol/l). Connective tissue was removed and aortas were cut into 2-mm segments. The aortic segments were transferred into scintillation vials containing Krebs-HEPES buffer with 100 µmol/l L-012 and were incubated for 5 min. Chemiluminescence was then assessed over 15 min in a scintillation counter (Lumat LB 9501, Berthold, Bad Wildbad, Germany) in 1 min intervals. The vessel segments were then dried and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per mg aortic tissue.

As a second method, the superoxide dismutase (SOD)–inhibitable cytochrome C reduction assay was used. Two aortic ring sections (2 mm) were placed in buffer containing 145 mmol/l NaCl, 4.86 mmol/l KCl, 5.7 mmol/l NaH\(_2\)PO\(_4\), 0.54 mmol/l CaCl\(_2\), 1.22 mmol/l MgSO\(_4\), 5.5 mmol/l glucose, 0.1 mmol/l deferoxamine mesylate, and 1 U/µl catalase. Cytochrome C (50 µmol/l; Sigma) was then added, and the samples were incubated at 37°C for 60 minutes with and without SOD (125 U/ml). Cytochrome C reduction was calculated using absorbance at 550 nm corrected for background readings at 540 and 560 nm. Superoxide production was
quantified in nanomoles per mg aortic tissue from the difference between absorbance with or without SOD, as described previously\textsuperscript{3,4}.

To assess vascular superoxide production in situ, dihydroethidium (DHE) fluorescence of aortic tissue sections was used. Aortas were prepared as described above and 4-mm segments were embedded in Tissue Tek OCT embedding medium (Miles Laboratories), snap-frozen, and stored at -80°C. Samples were sectioned on a Leica cryostat (10 µm) and placed on glass slides. Krebs-HEPES buffer containing 2 µmol/l DHE was topically applied to each tissue section and sections were incubated in a dark humidified chamber at 37°C for 30 min. In situ production of superoxide was visualized by fluorescence microscopy. Paired aortas from active and inactive mice were processed in parallel, and images were acquired with identical acquisition parameters and were stored digitally.

**Measurement of NADPH oxidase activity**

NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay in buffer B containing phosphate 50 mmol/l (pH 7.0), EGTA 1 mmol/l, protease inhibitors (Complete\textsuperscript{®}, Roche), sucrose 150 mmol/l, lucigenin 0.005 mmol/l, and NADPH 0.1 mmol/l, as described\textsuperscript{5}. Tissue was mechanically lysed using a glass/teflon potter in ice-cold buffer B, lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/ml. 100 µl aliquots of the protein sample were measured over 10 min in quadruplicates using NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501) in 1 min intervals.

**Western Blotting**

For preparation of membrane and cytosolic proteins, aortic tissue was homogenized in PBS and span 5 min at 1000 rpm (4°C). The pellet was dissolved in 1x Reporter Lysis Buffer (Promega) and underwent 3 freeze-thaw cycles at -80°C for 15 min before dilution in 100 mmol/l Tris-HCl, 5 mmol/l MgCl\textsubscript{2}, 0.6 mmol/l EDTA, and sonication. After spinning for 10
min at 3000 rpm, the supernatant was ultracentrifuged for 45 min at 25,000 rpm (4°C). The resulting pellet contained membrane proteins, the supernatant cytosolic proteins. Immunoblotting was performed using rac1 (clone 23A8, Biomol 05-389) mouse monoclonal antibody (1:250 dilution), p47phox (H195, Santa Cruz sc-14015) rabbit polyclonal (1:250) and p67phox (H300, Santa Cruz sc-15342) rabbit polyclonal (1:250) antibodies. β-Actin (1:250; Santa Cruz) was used to control for equal protein loading. Immunodetection was accomplished using the corresponding secondary antibodies and the enhanced chemiluminescence kit (Amersham).

**Rac1 GST-PAK Pull-Down Assay**

A glutathione-S-transferase (GST)-PAK-CD (PAK-CRIB domain) fusion protein, containing the rac1 binding region from human PAK1B was used to determine rac1 activity as described. Escherichia coli transformed with the GST-PAK-CD construct were grown at 37°C to an absorbance of 0.3. Expression of recombinant protein was induced by addition of 0.1 mmol/l isopropylthiogalactoside for 2 h. Cells were harvested, resuspended in lysis buffer (50 mmol/l Tris-HCl, pH 8, 2 mmol/l MgCl₂, 0.2 mmol/l Na₂SO₄, 10% glycerol, 20% sucrose, 2 mmol/l dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml aprotinin), and then sonicated. Cell lysates were centrifuged at 4°C for 20 min at 45,000×g, and the supernatant was incubated with glutathione-coupled Sepharose 4B beads (Amersham) for 30 min at 4°C. Protein bound to the beads was washed three times in lysis buffer, and the amount of bound fusion protein was estimated using Coomassie-stained SDS gels.

Aortic tissue was homogenized and resuspended in lysis buffer (Tris-HCl 50 mmol/l (pH 7.4), NaCl 100 mmol/l, MgCl₂ 2 mmol/l, benzamidine 1 mmol/l, NP-40 1%, glycerol 10%; leupeptin, pepstatin, and aprotinin 1 µg/ml, respectively), and then centrifuged for 5 min at 21,000g at 4°C. Aliquots were taken from the supernatant to compare protein amounts. Equal amounts of supernatant protein were incubated with the bacterially produced GST-PAK-CD
fusion protein bound to glutathione-coupled Sepharose beads at 4°C for 30 min. The beads and proteins bound to the fusion protein were washed three times with lysis buffer, eluted in Laemmli sample buffer (60 mmol/l Tris (pH 6.8), 2% sodium dodecylsulfate, 10% glycerin, 0.1% bromphenol blue), and then analyzed for bound rac1 molecules by Western blotting.

**mRNA isolation and polymerase chain reactions**

RNA from aortic homogenates was isolated with RNA-clean. 1 µg of the isolated total RNA was reverse transcribed using random primers and MMLV reverse transcriptase for 60 min at 42°C and 10 min at 75°C. The single stranded cDNA was amplified by polymerase chain reactions. Sequences for sense (S) and antisense (A) primers, PCR conditions and cycle counts were:

- **p22phox**: S: 5’-GAC-GCT-TCA-CGC-AGT-GGT-AC; A: 5’-CAC-GAC-CTC-ATC-TGT-CAC-TGG; 94°C, 1 min; 65°C, 1 min; 72°C, 1.5 min, 40 cycles;  
- **p67phox**: S: 5’-AGA-CAC-CTT-GAA-CTA-CCA-TCC; A: 5’-CTG-CTC-TTC-TGC-TTT-CTT-CC; 94°C, 1 min; 60°C, 1 min; 72°C, 1.5 min; 30 cycles;  
- **p47phox**: S: 5’-CGA-AAG-AGC-CTG-AGA-CAT-ACC; A: 5’-ATA-TCC-CCT-TTC-CTG-ACC-ACC; 94°C, 1 min; 65°C, 1 min; 72°C, 1.5 min; 30 cycles;  
- **nox4**: S: 5’-CAT-TTG-GCT-GCT-CCT-AAA-CG; A: 5’-AAC-AAA-CCA-CCT-GAA-ACA-TGC; 94°C, 1 min; 56°C, 1 min; 72°C, 1.5 min; 40 cycles;  
- **gp91phox**: S: 5’-CCT-ATG-ACT-TGG-AAA-TGG-AT; A: 5’-CAG-AGC-CAG-TAG-AAG-TAG-AT; 94°C, 30 s; 58°C, 30 s; 72°C, 45 s; 40 cycles;  
- **nox1**: S: 5’-TGT-GCC-GAC-CAC-ACT-CAA-CCT-GAA-GGA-ACA-TGC; 94°C, 1 min; 56°C, 1 min; 72°C, 1.5 min; 45 cycles. mRNA expression was normalized to expression of the housekeeping gene GAPDH (S: 5’-ACC-ACA-GTC-CAT-GCC-ATC-AC; A: 5’-TCC-ACC-ACC-TCT-GTG-CTG-CA; 94°C, 30 s; 55°C, 45 s; 72°C 45 s; 23 cycles).
Aortic ring preparations and tension recording

After excision of the descending aorta, the vessel was immersed in chilled buffer containing NaCl 118.0 mmol/l, CaCl₂ 2.5 mmol/l, KCl 4.73 mmol/l, MgCl₂ 1.2 mmol/l, KH₂PO₄ 1.2 mmol/l, NaHCO₃ 25.0 mmol/l, NaEDTA 0.026 mmol/l, D(+)-Glucose 5.5 mmol/l, pH 7.4. Adventitial tissue was carefully removed. 2-mm rings were mounted in organ baths filled with the above described buffer (37°C; continuously aerated with 95% O₂ and 5% CO₂), were attached to a force transducer, and isometric tension was recorded. The vessel segments were gradually stretched over 60 min to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for further 30 min. Drugs were added in increasing concentrations in order to obtain cumulative concentration-response curves: phenylephrine 1 nmol/l-10 µmol/l, carbachol 10 nmol/l-100 µmol/l, and nitroglycerin 1 nmol/l-10 µmol/l. The drug concentration was increased when vasoconstriction or relaxation was completed. Drugs were washed out before the next substance was added.

Staining of atherosclerotic lesions and morphometric analysis

The hearts with the ascending aorta were embedded in Tissue Tek O.C.T. embedding medium (Miles), snap frozen and stored at –80°C. Samples were sectioned on a Leica cryostat (10 µm), starting at the apex and through the aortic valve area into the ascending aorta, and were placed on slides. At least 25 consecutive sections per animal were used for analysis. For the detection of atherosclerotic lesions, the sections were fixed with 3.7% formaldehyde for 1h, rinsed with deionised water, stained with oil red O working solution (0.5%) for 30 min, and were rinsed again. For morphometric analysis, hematoxylin staining was performed according to standard protocols. All sections were examined under a Nikon E600 microscope. Lucia Measurement Version 4.6 software was used to measure lipid-staining area and total area of the histological sections.
**Immunohistochemistry**

For immunohistochemical analysis, aortic samples were sectioned on a Leica cryostat (7 µm) and placed on poly-L-lysine (Sigma) coated slides. Cryosections were assessed for the endothelial cell marker von Willebrand factor (vWF) (rabbit polyclonal antibody, clone A0082, 1:100, Dako) and the NADPH oxidase subunits p47phox (rabbit polyclonal antibody, sc-14015, 1:50, Santa Cruz) and p67phox (rabbit polyclonal antibody, sc-15342, 1:50, Santa Cruz). Cryosections were postfixed in 4% paraformaldehyde for 2 min and preincubated with 0.5% Igpal for 10 min and 0.5% normal goat serum (Sigma) for 30 min. The primary antibody was applied for 1-2 h at room temperature or at 4°C over-night. For light microscopy, a peroxidase-conjugated anti-rabbit secondary antibody (1:100, 45 min, Sigma) with DAB staining (Dako) was used. For fluorescence microscopy, a TRITC-conjugated anti-rabbit secondary antibody (1:100, 45 min, Sigma) and fluorescent mounting medium (Dako) were used. Nuclear staining was performed using 4´,6-diamidino-2-phenylindole (Dapi, Linaris). Isotype-specific secondary antibodies (Santa Cruz) were used for negative controls. All sections were examined under a Nikon E600 microscope.

**NOS Assays**

Aortic eNOS mRNA expression was quantitated with the TaqMan PCR system (Abi Prism 7700 Sequence Detection System, PE Biosystems). Primers for amplification of eNOS were 5’-TTC-CGG-CTG-CCA-CCT-GAT-CCT-AA and 5’-AAC-ATA-TGT-CCT-TCA-AGG-CA. For quantification, eNOS mRNA expression was normalized to the expressed housekeeping gene 18S ribosomal RNA. For 18S the primers were 5’-TTG-ATT–AAG-TCC-CTG-CCC-TTT-GT and 5’-CGA-TCC-GAG-GGC-CTA-ACT-A.

eNOS Western blotting was performed using a murine monoclonal antibody to human eNOS (1:400 dilution, Transduction Laboratories, Lexington, KY). β-Actin was used to control for equal protein loading (Santa Cruz Actin H-196 polyclonal antibody, 1:250 dilution).
To assess NOS activity, mouse aortas were homogenized in 250 mmol/l Tris-HCl, pH 7.4, 10 mmol/l EDTA, 10 mmol/l EGTA. Lysates were pelleted (10 min, 13,000 rpm, 4°C) and supernatants were used for the assay. The total protein concentration in the supernatants was quantified and 10 µg of protein per each sample was used. NOS activity was determined by measuring the conversion of [3H]-arginine to [3H]-citrulline using the NOS assay kit from Calbiochem according to the manufacturer’s instructions. Rat cerebellum served as positive control. Lysates incubated with the eNOS inhibitor, L-NAME (1 mmol/l) served as blanks.

**Statistical analysis** Results are presented as mean±SEM. Unpaired Student’s t-test and ANOVA for multiple comparisons were employed where applicable. Post-hoc comparisons were performed with the Neuman-Keuls test. Values of p<0.05 were considered statistically significant.

**Reference List**


