Preoperative Atorvastatin Treatment in CABG Patients Rapidly Improves Vein Graft Redox State by Inhibition of Rac1 and NADPH-Oxidase Activity

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Background—Statins improve clinical outcome of patients with atherosclerosis, but their perioperative role in patients undergoing coronary artery bypass grafting (CABG) is unclear. We hypothesized that short-term treatment with atorvastatin before CABG would improve the redox state in saphenous vein grafts (SVGs), independently of low-density lipoprotein cholesterol (LDL)-lowering.

Methods and Results—In a randomized, double-blind controlled trial, 42 statin-naive patients undergoing elective CABG received atorvastatin 40 mg/d or placebo for 3 days before surgery. Circulating inflammatory markers and malondialdehyde (MDA) were measured before and after treatment. SVG segments were used to determine vascular superoxide (O$_2^-$) and Rac1 activation. For ex vivo studies, SVG segments from 24 patients were incubated for 6 hours with atorvastatin 0, 5, or 50 µmol/L. Oral atorvastatin reduced vascular basal and NADPH-stimulated O$_2^-$ in SVGs (P<0.05 for all versus placebo) and reduced plasma MDA (P<0.05), independently of LDL-lowering and of changes in inflammatory markers. In SVGs exposed to atorvastatin ex vivo, without exposure to LDL, basal and NADPH-stimulated O$_2^-$ were significantly reduced (P<0.01 for both concentrations versus 0 µmol/L) in association with a striking reduction in Rac1 activation and 1 membrane-bound Rac1 and p67phox subunit. The antioxidant effects of atorvastatin were reversed by mevalonate, implying a dependence on vascular HMG-CoA reductase inhibition.

Conclusions—Short-term treatment with atorvastatin 40 mg/d before CABG improves redox state in SVGs, by inhibiting vascular Rac1-mediated activation of NADPH-oxidase. These novel findings suggest that statin therapy should be maintained or initiated in patients undergoing CABG, independently of LDL levels.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT01013103.
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Key Words: statins ■ malondialdehyde ■ oxidative stress ■ inflammation ■ CRP ■ NADPH-oxidase ■ superoxide ■ coronary bypass grafting ■ Rac1

Evidence suggests that statin treatment reduces cardiovascular risk. $^1$ Initiation of statin therapy is recommended in patients with coronary artery disease (CAD) when low-density lipoprotein cholesterol (LDL) levels are >100 mg/dL, whereas reduction of LDL to <70 mg/dL is now considered reasonable. $^1$

Despite the clear benefit of statin treatment on clinical outcome of CAD patients with elevated LDL, it is still unclear whether preoperative treatment with statins is beneficial in patients with low LDL undergoing coronary artery bypass grafting (CABG) surgery. Initial observations suggested that preoperative statin treatment reduces postoperative mortality $^2$ and minimizes complications, $^3$ but these findings have not been consistently reproduced. $^4$ Recent evidence suggests that statin treatment after CABG inhibits saphenous vein (SV) graft disease, $^5$ suggesting that optimal preoperative statin treatment might be an important factor in determining the long term benefit of CABG. Thus, understanding the acute effects of preoperative statins on vein graft biology remains an important goal.
Statins have a number of pleiotropic actions, which include antiinflammatory and systemic antioxidant effects. Indeed, recent evidence suggested that atorvastatin treatment exerts a systemic antioxidant effect within the first 3 days of treatment initiation in patients with atherosclerosis. Experimental studies have demonstrated that statins also reduce vascular superoxide (O$_2^-$) generation in animal and cell culture models, but the exact mechanisms are still under investigation. Statin treatment improves endothelial function in mammary artery grafts, but the mechanistic effects of statins on vascular redox state in human bypass grafts, beyond those exerted through systemic changes in LDL and inflammation, remain uncertain.

We hypothesized that short-term preoperative treatment with atorvastatin may improve vascular redox state in SV grafts used in CABG, by acting directly on the vascular wall, independently of LDL-lowering or changes in systemic inflammation. We examined, for the first time in humans, the direct effect of atorvastatin on redox state in SV grafts and explored the mechanisms by which atorvastatin may regulate vascular O$_2^-$ generation.

### Methods

#### Study Population and Clinical Protocol

In the first part of the study, we examined whether short-term preoperative treatment with atorvastatin affects redox state in SV

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**Table. Demographic Characteristics and Operation Details of the Participants**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Atorvastatin (40 mg/day)</th>
<th>Ex Vivo Study</th>
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<td></td>
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<tr>
<td>No. of patients, n (male/female)</td>
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<td>21 (18/3)</td>
<td>24 (19/5)</td>
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\(n\) indicates no. of patients; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker. There was no significant difference in serum lipid changes between atorvastatin- or placebo-treated groups (as tested by ANOVA for repeated measures with time \(\times\) treatment interaction). NS indicates no significant difference between the 2 treatment groups; \(*P<0.05\) vs baseline. Continuous variables are expressed as means±SEM.
grafts used for CABG. A total of 332 patients undergoing elective CABG were screened to identify patients who were not currently taking statins, and 42 of these patients fulfilled the inclusion criteria and agreed to participate. Patients were then randomized to receive either atorvastatin 40 mg/d or placebo for 3 days preoperatively in a double-blind fashion.

Blood samples were obtained at baseline and on the morning before the operation to measure markers of systemic oxidative stress and low-grade inflammation. At the time of surgery, segments of SV grafts were obtained and transferred to the laboratory, as described previously (online-only Data Supplement). Exclusion criteria were any inflammatory, infective, liver, or renal disease, malignancy, or known intolerance to statins. Patients receiving nonsteroidal anti-inflammatory drugs, dietary supplements, or antioxidant vitamins or who had received any statin treatment during the last 3 months were also excluded.

In the second part of the study, we examined whether the effect of atorvastatin on vascular redox of SV grafts was independent of LDL-lowering and explored possible alternative mechanisms. To achieve that, we used an ex vivo system of human vessels, in which we exposed SV grafts directly to atorvastatin in an LDL-free environment. For these experiments, 24 additional patients with coronary artery disease undergoing elective CABG were recruited. Blood samples from these patients were obtained at the morning before CABG and samples of SV grafts were harvested and transferred to the laboratory for the ex vivo experiments.

Demographic characteristics of the patients are presented in the Table. The study protocol was approved by the local research ethics committee, and each patient gave written informed consent.

**Determination of Vascular Superoxide Production**

Vascular O$_2^-$ production was measured in fresh segments of intact SV by using lucigenin-enhanced (5 μmol/L) chemiluminescence (online-only Data Supplement). These measurements are closely correlated with measurements in vascular homogenates from the same vessels, as we have demonstrated previously.

**Ex Vivo Protocol**

To examine the direct effect of atorvastatin on vascular O$_2^-$ generation in SV grafts, SV segments obtained from 24 patients were incubated ex vivo for 6 hours, in an organ bath system containing oxygenated (95% O$_2$/5% CO$_2$) Krebs–Henseleit buffer at 37°C, as we have described previously. Three rings from the same SV segment were incubated with 0, 5, and 50 μmol/L atorvastatin (n=24) for 6 hours. Both resting and NADPH-stimulated vascular O$_2^-$ were estimated in all 3 rings from each patient at the end of the incubation period. To examine whether the effects of atorvastatin on SV grafts were mediated through direct inhibition of hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase in the vascular wall, we incubated segments of SV grafts from the same patients with 0, 5, and 50 μmol/L atorvastatin in the presence and absence of mevalonate 200 μmol/L for 6 hours and measured resting and NADPH-stimulated vascular O$_2^-$ by lucigenin chemiluminescence at the end of the incubation period.

**Blood Sampling and Biochemical Measurements**

Venous blood samples were taken after a 12-hour fast at baseline and at the morning before the operation. After centrifugation at 2000g at 4°C for 15 minutes, plasma or serum was collected and stored at −80°C until assayed.

**Measurements of Plasma Malonyldialdehyde Levels**

Systemic lipid peroxidation was quantified by measuring plasma malonyldialdehyde (MDA) levels (online-only Data Supplement).

**Measurement of Systemic Inflammatory Markers**

Plasma high-sensitivity C-reactive protein (hsCRP) was measured by particle-enhanced immunonephelometry (N Latex, Dade-Behring, Marburg, Germany). Serum interleukin (IL)-6 and soluble vascular cell adhesion molecule (sVCAM)-1 were determined by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany).

**Measurements of Serum Lipid Levels**

Serum total cholesterol, LDL, high-density lipoprotein cholesterol (HDL), and triglycerides were measured by using a chromatographic enzymatic method in a Technicon automatic analyzer RA-1000 (Dade Behring).

**Measurement of Vascular Rac1 Activation**

Rac1 activation was evaluated by an affinity precipitation assay using the PAK1-PBD–conjugated glutathione agarose beads according to the instructions of the manufacturer (Millipore, Temecula, Calif) (online-only Data Supplement). Rac1 activation was defined by the ratio GTP-Rac1/total Rac1, as appropriate.

**Immunoblotting**

Immunoblotting was used to evaluate the Nox1, Nox2, and Nox4 protein level in vascular homogenates (online-only Data Supplement). The p47phox, p67phox, and Rac1 content in the membrane...
subfraction was compared in the presence or absence of atorvastatin treatment (ex vivo) (online-only Data Supplement).

**Oxidative Fluorescent Microtopography**

In situ superoxide production was determined in vessel cryosections with fluorescent dye dihydroethidium staining (online-only Data Supplement).

**Statistical Analysis**

All continuous variables were tested for normal distribution by using Kolmogorov–Smirnov test. Nonnormally distributed variables were log-transformed for analysis and are presented in a nonlogarithmic format as median [25th to 75th percentile]. Normally distributed variables are presented as means ± SEM. Power calculations based on our previous studies suggested that 20 subjects per group would be able to detect a 30% difference in vascular $O_2^-$ generation with $\alpha=0.05$ and power 90%.

Continuous variables between 2 independent groups were compared by using unpaired $t$ test, whereas categorical variables were compared by using $\chi^2$ test, as appropriate. The effect of treatment on plasma biomarkers was compared between the 2 groups by using 2-way ANOVA for repeated measures with time x treatment interaction. In the ex vivo experiments (where vascular segments from the same vessel were incubated with increasing concentrations of atorvastatin), we performed an overall repeated measures ANOVA, followed by Bonferroni post hoc correction for individual comparisons between groups. Correlations between continuous variables were assessed by using bivariate analysis, and Pearson’s coefficient was estimated. All statistical tests were performed by using SPSS v17.0, and $P<0.05$ was considered statistically significant.

**Results**

**Effects of Short-Term Oral Atorvastatin Treatment Before CABG**

There was no significant difference in demographic characteristics or baseline lipid levels between the study groups in the clinical trial (Table).

We first examined the effects of preoperative treatment with atorvastatin 40 mg/d for 3 days on vascular $O_2^-$ generation in SV grafts used for CABG. Atorvastatin significantly reduced basal and NADPH-stimulated vascular $O_2^-$ (Figure 1) and Rac1 activation in SV grafts (Figure 1), suggesting that $O_2^-$ generation in SV grafts occurred by suppressing Rac1-mediated NADPH-oxidase stimulation. Under basal conditions, nitric oxide synthase (NOS) inhibition caused a reduction of vascular $O_2^-$, suggesting that NOS was largely uncoupled in these vessels. As shown in Figure 1, atorvastatin reversed $\text{N}^2\text{-nitro-L-arginine methyl ester (L-NAME)}$–inhibitable $O_2^-$ generation, suggesting an improvement of NOS coupling in these SV grafts. However, short-term atorvastatin treatment had no impact on Nox1, Nox2, or Nox4 protein levels in these grafts (Figure 2). These findings were in line with the significant reduction of plasma MDA levels observed in the treated compared to placebo patients (Figure 3), indicating an effect on systemic oxidative stress.
Because systemic inflammation has a direct impact on vascular and systemic oxidative stress,\(^1\)\(^3\) and chronic statin treatment has been shown to have antiinflammatory properties,\(^1\) we examined whether atorvastatin exerts its effects on \(O_2^-\) generation in SV grafts by decreasing systemic low-grade inflammation. We found that atorvastatin had no impact on circulating IL-6, sVCAM-1, or hsCRP, indicating that 3 days of statin therapy is insufficient to induce a detectable effect on systemic inflammation (Figure 3). There was no association between basal \(O_2^-\) in SV grafts and either circulating hsCRP (\(r\) [95% CI] = 0.192 [-0.12 to 0.47]; \(P = 0.346\)), IL-6 (\(r\) [95% CI] = 0.265 [-0.04 to 0.53]; \(P = 0.172\)), or sVCAM-1 (\(r\) [95% CI] = -0.009 [-0.31 to 0.29]; \(P = 0.966\)) or between NADPH-stimulated \(O_2^-\) and hsCRP (\(r\) [95% CI] = 0.254 [-0.05 to 0.52]; \(P = 0.232\)), IL-6 (\(r\) [95% CI] = -0.021 [-0.32 to 0.29]; \(P = 0.920\)), or sVCAM-1 (\(r\) [95% CI] = -0.065 [-0.37 to 0.25]; \(P = 0.757\)). Similarly, there was no association between the change of MDA and the changes of hsCRP (\(r\) [95% CI] = -0.155 [-0.16 to 0.44]; \(P = 0.431\)), IL-6 (\(r\) [95% CI] = 0.052 [-0.26 to 0.35]; \(P = 0.790\)), or sVCAM-1 (\(r\) [95% CI] = -0.266 [-0.53 to 0.44]; \(P = 0.147\)) in the overall study population. These findings suggest that the rapid effect of atorvastatin on SV graft redox state is independent of changes in systemic inflammation.

**Figure 3.** Preoperative treatment with atorvastatin 40 mg/d for 3 days significantly reduced plasma malonyldialdehyde (MDA) levels (A) but failed to modify circulating levels of IL-6 (B), hsCRP (C), or sVCAM-1 (D). Values expressed as means±SEM. \(*P<0.05\) vs pretreatment or versus placebo (after ANOVA with time×treatment interaction).

**Effects of Atorvastatin on Saphenous Vein Graft Redox State Ex Vivo**

To further examine whether atorvastatin exerts its effects on \(O_2^-\) generation in SV grafts directly, independently of LDL-lowering, we used an ex vivo system, in which SV graft segments (n=24) were incubated with atorvastatin 0, 5, or 50 μmol/L for 6 hours. We observed that both concentrations of atorvastatin induced a significant reduction of resting and NADPH-stimulated \(O_2^-\) generation in SV grafts (Figure 4). This effect was independent of resting or NADPH-stimulated \(O_2^-\) at baseline. The effect on intact vessels was confirmed in homogenates of SV grafts (n=7), where \(O_2^-\) generation was reduced from 4.2[3.2 to 7.2] relative light units (RLU)/sec per microgram of protein in 0 μmol/L to 2.6[1.7 to 3.2] RLU/sec per microgram of protein after incubation with 5 μmol/L atorvastatin and 2.7[2.2 to 3.9] RLU/sec per microgram of protein after incubation with 50 μmol/L atorvastatin for 6 hours (n=7, \(P<0.05\) for both versus 0 μmol/L). Similarly, NADPH-stimulated \(O_2^-\) generation was reduced

**Figure 4.** Ex vivo incubation of SV grafts (n=16) with atorvastatin for 6 hours reduced both resting (A) and NADPH-stimulated (B) superoxide (\(O_2^-\)) generation. Moreover, atorvastatin incubation reduced activated Rac1 (expressed as the ratio of GTP-Rac1/total Rac1) in these grafts (C). Values are expressed as median [25th to 75th percentile], range (A and B), or means±SEM (C). Overall repeated measures comparison revealed a significant effect of treatment in all readouts (\(P<0.01\) for all figure panels). \(*P<0.01\) for individual comparisons with control (0 μmol/L). Shown is an example of SV segments obtained from 2 patients indicated as SV1 and SV2, respectively. + indicates positive control; −, negative control.
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Figure 5. Ex vivo incubation of SV grafts with atorvastatin for 6 hours reduced membrane-bound Rac1 and p67\textsubscript{phox} subunit of NADPH-oxidase (B). Values are expressed as means±SEM. A, Shown is an example of SV segments obtained from 3 patients indicated as SV1, SV2, and SV3, respectively.

Discussion

In the present study, we demonstrate that preoperative treatment with atorvastatin 40 mg/d for 3 days reduces O$_2^{\text{--}}$ generation in SV grafts used for CABG surgery. We also
demonstrate that atorvastatin exerts its effects by directly inhibiting HMG-CoA reductase in the vascular wall, independently of LDL-lowering and systemic inflammation. Atorvastatin suppresses Rac1-mediated activation of vascular NADPH-oxidase, although it also reduces membrane-bound p67<sub>phox</sub> subunit of the enzyme. We also demonstrate that atorvastatin directly improves eNOS-coupling in these grafts, suggesting an additional mechanism by which statins exert their vascular antioxidant effects. These novel findings indicate that even short-term statin treatment during the preoperative period may have an important beneficial effect on SV graft biology and redox state.

Recent evidence suggests that statins reduce cardiovascular risk even in low-risk subjects with LDL concentrations of <130 mg/dL, especially in the presence of high background inflammatory status. In addition, evidence suggests that preoperative statin treatment reduces mortality post CABG<sup>2</sup> and minimizes postoperative complications,<sup>3</sup> although a recent retrospective study<sup>5</sup> suggested that statins may also improve long-term SV grafts patency, a finding that requires further investigation. It is, therefore, important to understand how preoperative treatment with statins affects the biology of SV grafts, including those patients with relatively low LDL.

Vascular O<sub>2</sub><sup>−</sup> is a critical feature regulating vascular homeostasis, whereas NADPH-oxidase, a major source of O<sub>2</sub><sup>−</sup> in the vascular wall,<sup>14</sup> could potentially be a therapeutic target in atherosclerosis.<sup>15</sup> Evidence suggests that short-term treatment with atorvastatin modifies systemic oxidative stress,<sup>2</sup> but the mechanisms mediating this effect in humans are unclear. Experimental studies have demonstrated that statins reduce endothelium-derived O<sub>2</sub><sup>−</sup> in an animal model<sup>8</sup> and inhibit intimal hyperplasia in rabbit vein grafts.<sup>16</sup> These effects had been suggested to be partly attributable to improvement of eNOS activity<sup>17</sup> or suppression of Rac1-mediated activation of NADPH-oxidase in these models,<sup>8</sup> but the relevance of these observations to the acute vascular effects of statins in humans have remained unclear. In the present study, we now demonstrate that short-term treatment with atorvastatin 40 mg/d for 3 days before CABG suppresses O<sub>2</sub><sup>−</sup> generation in SV grafts in statin-naïve patients. We also demonstrate that this effect is attributable to a decrease of NADPH-oxidase activity in the vascular wall. Although short-term atorvastatin treatment had no effect of the expression of Nox1, Nox2, or Nox4 complexes of NADPH-oxidase in these grafts, we demonstrate that it decreases the activation of Rac1, a GTPase with a known regulatory role in NADPH-oxidase activation.<sup>14</sup> Atorvastatin also reduced plasma MDA, indicating a reduction of systemic oxidative stress, an effect that was independent of LDL changes.

Systemic inflammation is a stimuli for O<sub>2</sub><sup>−</sup> generation in the vascular wall,<sup>13</sup> whereas statins exert a global antiinflammatory effect.<sup>1</sup> It is, therefore, possible that atorvastatin exerts its antioxidant effects on O<sub>2</sub><sup>−</sup> generation in SV grafts by modulating systemic low-grade inflammation. However, in the present study, circulating IL-6, hsCRP, and sVCAM-1 remained unchanged after treatment with atorvastatin for 3 days and were unrelated to vascular O<sub>2</sub><sup>−</sup>. These findings are consistent with previous reports indicating that short-term treatment (<5 days) with atorvastatin does not affect systemic low-grade inflammation,<sup>18</sup> confirming that systemic inflammation did not mediate the effect of atorvastatin on SV graft redox state in the present study.

To further examine whether the effect of atorvastatin on vascular redox was independent of LDL-lowering or systemic low-grade inflammation, we used an ex vivo model of human SV grafts, and we demonstrated that atorvastatin, by acting directly on the vascular wall, suppresses Rac1 activation and reduces membrane-bound Rac1 and p67<sub>phox</sub> subunit, resulting into a reduction of resting and NADPH-stimulated O<sub>2</sub><sup>−</sup> in SV grafts. This effect was reversed by mevalonate, suggesting that inhibition of HMG-CoA reductase is the main mechanism by which atorvastatin exerts its effects on vascular redox state.

Furthermore, NADPH-oxidase, the uncoupled eNOS, is another major source of O<sub>2</sub><sup>−</sup> in the vascular wall<sup>19</sup>; therefore, recoupling of eNOS may be a rational therapeutic goal in SV grafts biology. In endothelial cell culture models, statins
improve eNOS-coupling and reduce cellular O$_2$$^{-}\cdot$ generation,\textsuperscript{20} but their effect on eNOS-coupling in human SV grafts is unknown. In the present study, we demonstrate that even short-term oral treatment with atorvastatin improves eNOS-coupling in human SV grafts. Moreover, we demonstrate that atorvastatin improves eNOS-coupling in these vessels by directly inhibiting HMG-CoA reductase in the vascular wall, in an ex vivo system of human SV grafts.

The results of the present study may also be extrapolated to other vascular beds. Indeed, although our study examined the impact of short-term statin treatment on the mechanisms regulating redox state in SV grafts of patients undergoing CABG, our results may also be applicable to arterial vascular beds. Short-term treatment with atorvastatin rapidly improves endothelial function in the brachial artery even within 24 hours,\textsuperscript{21} by mechanisms independent of lipid-lowering.\textsuperscript{6} This may also be mediated by an effect on Rac1 activation, modification of NADPH-oxidase activity, and improvement of eNOS-coupling, providing a possible explanation for the clinical benefits observed after short-term statin treatment of patients with acute coronary events.\textsuperscript{22} However, further studies focused on human arterial tissue are required to investigate this hypothesis.

In conclusion, we demonstrate, for the first time in humans, that preoperative treatment of statin-naïve patients with atorvastatin 40 mg/d for 3 days inhibits HMG-CoA reductase directly in the vascular wall, independently of LDL-lowering or systemic low-grade inflammation. This results into a reduction of O$_2$$^{-}\cdot$ generation in SV grafts by modifying Rac1-mediated activation of NADPH oxidase, as well as by modifying eNOS-coupling. These novel findings may have major clinical implications, because they support the notion that even in patients with relatively low LDL, preoperative treatment with statins has direct beneficial effects on the biology of SV grafts used in CABG and may have an impact on the patency of these grafts.

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Disclosures

None.

References

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Online Supplemental Data

Supplemental Methods

Vessel Harvesting

Samples of SV grafts were obtained at the time of CABG as we have described previously. \(^1,2\) Briefly, samples of SV grafts were harvested by using a “no touch” technique and transferred into oxygenated (95%O\(_2\) / 5% CO\(_2\)) ice-cold Krebs Hensleit Buffer, before any manipulation or dilatation of the graft by the surgeon. No vasodilator agents were used. Adventitia was removed from these vessels, while the lumen was flushed gently by using an insulin syringe to remove blood. Vascular rings of SV grafts (~3-4mm length) were snap frozen in the operating room. Additional SV graft rings of appropriate size were transferred to the laboratory within 30 minutes, in ice-cold Krebs Henseleit buffer. In the lab, these rings were either used immediately for luminometry experiments (for the oral study) or were used for the ex vivo experiments.

Determination of Vascular Superoxide Production

Vascular O\(_2^-\) production was measured in fresh segments of intact SV by using lucigenin (5 μmol/L)-enhanced chemiluminescence, as described previously. \(^1,3\) Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O\(_2\)/5% CO\(_2\)) Krebs-HEPES buffer (pH 7.4) at 37°C. Lucigenin-enhanced chemiluminescence was measured with low-concentration lucigenin (5 μmol/L). \(^3\) NADPH-stimulated O\(_2^-\), a measure of NADPH-oxidase activity, was estimated after adding 100 μmol/L of NADPH, as described previously. \(^1\) These measurements are closely correlated with measurements in vascular homogenates from the same vessels, as we have demonstrated in the past. \(^4\)
The contribution of uncoupled nitric oxide synthase (NOS) to vascular O$_2^-$ production was evaluated by estimating the difference in O$_2^-$ after 20 minutes of incubation with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L).\textsuperscript{1}

**Oxidative Fluorescent Microtopography**

In situ superoxide production was determined in vessel cryosections with fluorescent dye dihydroethidium (DHE) staining as we have previously described.\textsuperscript{5} Cryosections (30 µm) of SV grafts from the *ex vivo* study, were incubated with DHE (2 µmol/L) in Krebs-HEPES buffer 37$^\circ$ C for 5 minutes, with or without polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD) (500 µmol/L). Fluorescent images of the endothelium (Zeiss LSM 510 META laser scanning confocal microscope, Carl Zeiss, Inc, Oberkochen, Germany) were obtained from each vessel quadrant. In each case, segments of vessel rings from the same SV graft incubated with 0, 5 and 50 µmol/L atorvastatin were analyzed in parallel with identical imaging parameters. DHE fluorescence was quantified by automated image analysis with Image-Pro Plus software (Media Cybernetics, Bethesda, Md).

**Measurements of Plasma Malonyldialdehyde Levels**

Systemic lipid peroxidation was quantified by measuring malonyldialdehyde (MDA) levels in plasma.\textsuperscript{6} Briefly, blood samples were collected into tubes containing EDTA as anticoagulant, centrifuged and appropriately stored at −80$^\circ$C until assayed. The determination of MDA was based upon its reaction with thiobarbituric acid (TBA). Briefly, 50µL of plasma/standards were placed into tubes containing TBA dissolved in
acetic acid. Samples were heated at 95-100°C for 1 hour and thereafter they were cooled and HCl and subsequently butanol were introduced into the mixture. After centrifugation at 3500 rpm for 10 min butanol phase was removed. The fluorescence of the butanol extract was measured by a Perkin-Elmer fluorometer as previously described.6

Measurement of Vascular Rac1 Activation

Rac1 activation was evaluated by an affinity precipitation assay using the PAK1-PBD conjugated glutathione agarose beads according to the manufacturer’s instructions (Millipore, Temecula, USA). Briefly, human SV samples were homogenized on ice in 500 µL of lysis buffer. Pull-down of GTP-bound Rac1 was performed by incubating tissue lysates (100 µg) with GST fusion protein corresponding to the p21-binding domain of PAK-1 bound to glutathione-agarose for 1 h at 4 °C. The beads were washed three times with lysis buffer, and the protein bound to the beads was eluted with Laemmli buffer and analyzed for the amount of GTP-bound Rac1 by immunoblotting using a Rac1 monoclonal antibody. Rac1 activation was defined by the ratio GTP-Rac1/total Rac1, as appropriate.

Extraction of membrane-bound proteins by subcellular fractionation for the detection of the translocated cytosolic subunits of NADPH oxidase.

Differential centrifugation was used for isolation of membrane proteins as described elsewhere7. Briefly, vascular tissue was homogenized in ice-cold HEPES buffer (20 mM HEPES, 150 mM, NaCl, and 1 mM EDTA, pH 7.4), to which the protease inhibitor cocktail (Roche, UK) was added just before homogenization. Cell nuclei and
unbroken cells were removed by centrifugation of the homogenates at 2,800 g at 4°C for 20 min. The supernatants were further centrifuged at 100,000 g for 60 min at 4°C to separate the membrane proteins. The pellets, which contained the membrane protein fraction, were resuspended in HEPES buffer containing 1% Triton for 20 min on ice and used for assay of membrane-translocated p47\textsuperscript{phox}, p67\textsuperscript{phox} and Rac-1 proteins by Western immunobloting.

**Western immunoblotting of NOX1, NOX2, NOX4 and membrane-translocated p47\textsuperscript{phox}, p67\textsuperscript{phox} and Rac-1 proteins.**

To detect protein expression of NOX1, NOX2 and NOX4, vascular tissue samples were homogenized for 30 seconds using a pre-cooled electric homogenizer Polytron in 220 µl of lysis buffer (Invitrogen, UK) containing a protease inhibitor cocktail (Roche, UK). Homogenates were spun at 13,000 rpm for 10 minutes, at 4 °C. For the translocation experiments, the sample proteins were extracted as described above.

The protein concentration of the supernatants for both experiments was then measured using the BCA\textsuperscript{TM} Protein Assay kit (Pierce, UK). Protein lysates were separated on 4-12% gradient SDS-NuPAGE gel (Invitrogen, UK), and proteins transferred to polyvinylidene difluoride membranes (Amersham, UK Ltd.), and followed by blocking with 5% skimmed milk. The membranes were incubated with anti-NOX1 antibody (Abcam), anti-NOX2 (a kind gift from Dr Mark Quinn, Montana, USA), anti-NOX4 (a kind gift from Prof. Ajay Shah, London, UK), anti-p47\textsuperscript{phox} antibody (Upstate Biotechnology), anti-p67\textsuperscript{phox} antibody (Upstate Biotechnology) and anti-Rac-1 antibody (Millipore, USA) respectively, at room temperature for 1 hour. Immunodetection of the
primary antibodies was performed with horseradish-peroxidase-conjugated secondary antibodies (Promega). Immunological signal was visualized with anti-IgG-horseradish-peroxidase and enhanced chemifluorescence (Amersham Bioscience UK Ltd.) and quantified in relation to the house-keeping protein, GAPDH (Santa Cruz Santa Biotechnology, USA).

Supplemental References


