Simvastatin Prevents Vascular Hyporeactivity During Inflammation

Johannes Pleiner, MD; Georg Schaller, MD; Friedrich Mittermayer, MD; Stefan Zorn, MS; Claudia Marsik, MD; Stefan Polterauer, MS; Stylianos Kapiotis, MD; Michael Wolzt, MD

Background—There is growing evidence that statins exert anti-inflammatory and antioxidative vascular actions that are independent of lipid lowering. We tested whether hyporeactivity to the endothelium-dependent vasodilator acetylcholine (ACh) and the vasoconstrictor norepinephrine (NE) during acute experimental inflammation could be prevented by simvastatin.

Methods and Results—In a randomized, placebo-controlled, parallel group study, forearm blood flow (FBF) responses to NE, ACh, and the endothelium-independent vasodilator nitroglycerin (NTG) were assessed at baseline, after 4 days of simvastatin 80 mg PO or placebo treatment, and during Escherichia coli endotoxin (lipopolysaccharide [LPS])–induced inflammation in 20 healthy volunteers. Additionally, markers of inflammation and neutrophil oxidative burst were assessed. Simvastatin and placebo had no effect on FBF or oxidative/inflammatory markers. LPS administration decreased the responses of FBF to NE by 43% (P<0.05) and decreased responses to ACh by 48% (P<0.05) but did not decrease FBF responses to NTG. Simvastatin completely preserved responses to NE and to ACh. The LPS-induced increases in neutrophil oxidative burst and plasma tumor necrosis factor-α concentrations were mitigated by simvastatin (P<0.05 versus placebo).

Conclusions—This study demonstrates potent vasoprotective properties of high-dose simvastatin during endotoxemia that may be useful for patients with acute systemic inflammation and associated vascular hyporeactivity. (Circulation. 2004;110:3349-3354.)

Key Words: inflammation • endothelium • vasoconstriction • statins

The beneficial effect of statins to prevent cardiovascular events is well established in patients with hypercholesterolemia.1,2 This salutary outcome was mainly attributed to decreased circulating cholesterol concentrations. However, several studies have demonstrated that statins can exert a number of vascular actions that are independent of lipid lowering. Statins may increase endothelial nitric oxide synthase activity and expression3 and exert potent anti-inflammatory and antioxidative properties,4–6 which may translate into improved vascular function.

In view of the growing evidence that inflammation processes are present in atherosclerosis and that markers of systemic inflammation such as C-reactive protein are associated with increased cardiovascular morbidity and mortality,7 therapies to reduce inflammation might be beneficial in atherosclerosis and its sequelae. Statins were shown to reduce concentrations of high-sensitivity C-reactive protein8 and soluble CD40 ligand9 in long-term studies. An acute anti-inflammatory effect of statin therapy was seen in patients with acute coronary syndromes after 5 days.10 Short-term simvastatin therapy recently has been reported to reduce mortality in 2 different animal models of sepsis.11,12

We have previously demonstrated that acute systemic inflammation, as induced by low doses of Escherichia coli lipopolysaccharide (LPS), results in marked endothelial dysfunction and impaired responsiveness to vasoconstrictors,13 which is associated with oxidative stress.14,15 To characterize the vasoprotective properties of simvastatin during inflammation, we studied whether LPS-induced hyporeactivity to the endothelium-dependent vasodilator acetylcholine (ACh) and the vasoconstrictor norepinephrine (NE) in the human forearm vasculature could be prevented by short-term treatment with high therapeutic doses of simvastatin.

Methods

The study protocol was approved by the Ethics Committee of the University of Vienna and complies with the Declaration of Helsinki, including current revisions, and the Good Clinical Practice Guidelines for clinical trials.

Study Population

After giving informed consent, 22 healthy male subjects aged between 20 and 40 years were enrolled in this randomized, double-blind, parallel group study. Two subjects were withdrawn because of...
TABLE 1. Baseline Characteristics of Subjects Before Simvastatin/Placebo Treatment

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin, n=10</th>
<th>Placebo, n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>28±2</td>
<td>26±1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76±3</td>
<td>77±5</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.8±1.0</td>
<td>23.4±1.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>125±3</td>
<td>127±6</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>69±3</td>
<td>68±4</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>169±13</td>
<td>173±13</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>49±4</td>
<td>51±3</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>100±11</td>
<td>104±12</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>102±19</td>
<td>91±10</td>
</tr>
</tbody>
</table>

Data are mean±SEM. No significant differences were observed between groups.

difficulties with arterial puncture. All subjects were given a complete health examination (including physical examination, ECG, and laboratory screening) ≤14 days before the first study day. All subjects were nonsmokers and had no history or signs of arterial hypertension, major disorders in lipid metabolism, or other cardiovascular risk factors (Table 1). No additional medications, including over-the-counter drugs, besides the study medication were allowed from 3 weeks before screening until the study was completed.

Study Protocol
Subjects were randomized to receive either 80 mg of simvastatin or placebo for 4 days as a single oral dose in the morning (Figure 1). Baseline forearm blood flow (FBF) measurements and dose-response curves to intra-arterial NE, ACh, and glycerclyl trinitrate (nitroglycerin/NTG) were performed before first administration of simvastatin or placebo. Supervised drug intake commenced directly after FBF studies and was continued for the 2 subsequent days. On the fourth day, FBF measurements and dose-response curves were reassessed, and subjects received their last dose of simvastatin or placebo. Thereafter, LPS 20 IU/kg body weight (dose corresponding to 2 nmol/kg; National Reference Endotoxin, E coli, United States Pharmacopeia Convention Inc, Rockville, Md) was administered intravenously as a bolus infusion to induce acute inflammation. Injection of LPS to humans has been established by other groups and at our institution as a model to study cardiovascular responses to acute endotoxin (TNF-α) and IL-1β that were quantified by commercially available ELISA kits (R&D Systems). Oxidative burst of neutrophils was assessed by a dihydrodihydramine flow cytometric assay, as introduced by Bass et al.19 Briefly, whole blood was mixed with PBS or N-formyl-methionyl-leucyl-phenylalanine (fMLP) solution according to the manufacturer’s instructions and kit (Phagoburst, Orpegen). Fluorescence intensity of neutrophils, which is directly proportional to the average superoxide generation within the reacting cells, was measured by flow cytometry (FACScalibur flow cytometer, Becton Dickinson). Results are presented as percentage of spontaneously activated cells or the binding index, which was computed by the following equation: (percentage of positive cells×mean fluorescence intensity)/100.20 Cell counts were obtained with a cell counter (Sysmex, Milton Keynes).

FBF Measurements
FBF was measured as described previously.13,21 Briefly, strain gauges were placed on the forearms and connected to plethysmographs (EC-6, DE Hokanson) to measure changes in forearm volume in response to inflation of venous congesting cuffs. Bilateral plethysmography was used, with drug effects expressed as the ratio of blood flow in the intervention to that in the control arm.13,22 where baseline ratio was defined as 100%. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation to the hands. Flow measurements were recorded for 9 seconds at 30-second intervals during drug infusion. Traces were analyzed with NIVP3 software (version 5.25, Hokanson). Forearm vascular resistance was calculated by dividing mean arterial pressure by FBF.

A fine-bore needle (27-gauge needle; Sterican, B. Braun) was inserted into the brachial artery of the nondominant arm for intra-arterial infusion of vasoactive substances. After a 20-minute resting period, baseline FBF measurements were made in response to increasing intra-arterial doses of NE (60, 120, and 240 pmol/min; Arterenol, Aventis), the endothelium-dependent vasodilator ACh (25, 50, and 100 nmol/min; Clinalfa), and the endothelium-independent vasodilator NTG (4, 8, and 16 nmol/min; Perlinganit, Nycomed Imaging AS). NE was infused for 5 minutes per dose, whereas vasodilators were infused for 3 minutes. A washout period of 10 minutes established control blood flow between drugs under study.

Statistical Analysis
All data sets were tested for normal distribution by the Kolmogorov-Smirnov test. Data for white blood cell count, oxidative burst, markers of inflammation, and FBF were normally distributed. Data for temperature, pulse rate, and blood pressure were log-normally distributed and therefore log-transformed for comparisons, after which they tested correctly for normal distribution. Hemodynamics and laboratory parameters were expressed as absolute values. Comparisons before and after LPS within groups were done with t tests for independent samples. Comparisons between the simvastatin and placebo groups before and after LPS were made with respect to the difference in outcome measures with t tests for independent samples.

FBF was expressed as mL · min⁻¹ · 100 mL⁻¹ forearm volume. FBF responses to NE, ACh, and NTG were measured as described previously.18 Interleukin (IL)-6, E-selectin, tumor necrosis factor-α (TNF-α), and IL-1β were measured by commercially available ELISA kits (R&D Systems). Oxidative burst of neutrophils was assessed by a dihydrodihydramine flow cytometric assay, as introduced by Bass et al.19 Briefly, whole blood was mixed with PBS or N-formyl-methionyl-leucyl-phenylalanine (fMLP) solution according to the manufacturer’s instructions and kit (Phagoburst, Orpegen). Fluorescence intensity of neutrophils, which is directly proportional to the average superoxide generation within the reacting cells, was measured by flow cytometry (FACScalibur flow cytometer, Becton Dickinson). Results are presented as percentage of spontaneously activated cells or the binding index, which was computed by the following equation: (percentage of positive cells×mean fluorescence intensity)/100.20 Cell counts were obtained with a cell counter (Sysmex, Milton Keynes).

Markers of Inflammation and Oxidative Stress
Markers of inflammation and oxidative stress were measured in venous blood samples collected from subjects at baseline, 3 days after simvastatin/placebo, and 4 hours after LPS administration. Stable nitric oxide end products (nitrite and nitrate [NOx]) were measured as described previously.18 Interleukin (IL)-6, E-selectin, tumor necrosis factor-α (TNF-α), and IL-1β were quantified by commercially available ELISA kits (R&D Systems). Oxidative burst of neutrophils was assessed by a dihydrodihydramine flow cytometric assay, as introduced by Bass et al.19 Briefly, whole blood was mixed with PBS or N-formyl-methionyl-leucyl-phenylalanine (fMLP) solution according to the manufacturer’s instructions and kit (Phagoburst, Orpegen). Fluorescence intensity of neutrophils, which is directly proportional to the average superoxide generation within the reacting cells, was measured by flow cytometry (FACScalibur flow cytometer, Becton Dickinson). Results are presented as percentage of spontaneously activated cells or the binding index, which was computed by the following equation: (percentage of positive cells×mean fluorescence intensity)/100.20 Cell counts were obtained with a cell counter (Sysmex, Milton Keynes).

FIGURE 1. Flow chart of study. FBF measurements in response to increasing doses of NE, ACh, and NTG.
TABLE 2. Changes in Hemodynamic Parameters at Baseline and After Simvastatin/Placebo Treatment, Before and 4 Hours After Intravenous LPS Administration

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Before LPS</th>
<th>4 Hours After LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>67±4</td>
<td>70±3</td>
<td>89±3*</td>
</tr>
<tr>
<td>Placebo</td>
<td>71±4</td>
<td>63±2</td>
<td>91±3*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>88±2</td>
<td>90±3</td>
<td>90±4</td>
</tr>
<tr>
<td>Placebo</td>
<td>88±4</td>
<td>87±4</td>
<td>83±3</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>...</td>
<td>36.0±0.2</td>
<td>37.4±0.2*</td>
</tr>
<tr>
<td>Placebo</td>
<td>...</td>
<td>36.0±0.1</td>
<td>37.4±0.2*</td>
</tr>
<tr>
<td>Forearm blood flow, mL·min⁻¹·100 mL⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>5.6±0.9</td>
<td>6.5±0.7</td>
<td>6.7±1.3</td>
</tr>
<tr>
<td>Placebo</td>
<td>4.9±0.6</td>
<td>5.6±0.7</td>
<td>7.7±1.2*</td>
</tr>
<tr>
<td>Forearm vascular resistance, units</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>19.1±2.7</td>
<td>15.2±1.8</td>
<td>17.0±2.6</td>
</tr>
<tr>
<td>Placebo</td>
<td>21.0±3.2</td>
<td>18.6±3.0</td>
<td>12.1±2.1*</td>
</tr>
</tbody>
</table>

n=10 per group; data are mean±SEM. There was no significant difference between groups with respect to the difference of outcome measures before and after LPS (t test for independent samples).

*P<0.05 vs before LPS (t test for dependent samples).

Post hoc comparisons at individual infusion steps were performed by the Bonferroni test. The Statistica software package (release 5.0, StatSoft Inc) was used for all analyses. P<0.05 was considered significant. Values are presented as mean±SEM unless otherwise indicated.

Results

Study drugs were well tolerated, and no adverse events were reported. Subjects in the simvastatin and placebo groups were comparable with regard to physical and biochemical parameters at baseline (Table 1).

Systemic Hemodynamic and Humoral Effects

Simvastatin and placebo had no effects on systemic hemodynamics, baseline FBF, or forearm vascular resistance (Table 2). Simvastatin treatment resulted in a decrease in total cholesterol to 140±10 mg/dL and in LDL cholesterol to 81±9 mg/dL (P<0.05). After placebo, no significant changes were seen, but total cholesterol (175±13 mg/dL) and LDL (109±12 mg/dL) levels were higher than in the treatment group (P<0.05). HDL cholesterol and triglyceride levels were not significantly altered by simvastatin (P=0.11 and P=0.06, respectively) or placebo (P=0.51 and P=0.28, respectively). IL-6, E-selectin, TNF-α, IL-1β, NOx, PBS, and fMLP-induced oxidative burst were not changed by simvastatin or placebo (Table 3).

After LPS, the expected mild and transient flu-like symptoms occurred. In both treatment groups, LPS increased the white blood cell count after 4 hours (P<0.05; Table 3), which was paralleled by an increase in body temperature (P<0.05) and pulse rate (P<0.05) and a small reduction in blood pressure (Table 2). Mean FBF increased significantly in subjects receiving placebo (P<0.05; Table 2). Consequently, forearm vascular resistance decreased in the placebo group (P<0.05 versus baseline; Table 2). Hemodynamic parameters returned to baseline 8 hours after LPS (data not shown). NOx levels were unchanged after LPS. E-selectin, IL-6, and IL-1β concentrations increased significantly after LPS, with no differences between groups (Table 3). The increase in TNF-α levels, however, was significantly lower after simvastatin than after placebo (P<0.05; Table 3). The percentage of spontaneously activated neutrophils was significantly augmented after LPS in the placebo group but was completely prevented by simvastatin (P<0.05 between groups; Table 3). The binding index of fMLP-stimulated neutrophils was also higher in the placebo group (25.3±4.6) than in the simvastatin group after LPS (11.1±2.4; P<0.05).

FBF Studies

At baseline, intra-arterial ACh and NTG increased FBF (Figures 2 and 3), and NE induced the expected dose-dependent decrease in FBF to a similar extent in both groups (Figure 4). After simvastatin or placebo treatment, no significant changes in FBF responses to ACh, NTG, or NE were observed (Figures 2 through 4). LPS significantly reduced endothelium-dependent vasodilation to ACh in the placebo group (Figure 2; P<0.01 versus before LPS). Simvastatin prevented the LPS-induced impair-
ment of ACh-induced vasodilation (Figure 2 and Table 4; \(P<0.05\) versus placebo). Endothelium-independent vasodilation to NTG was not affected by LPS in subjects receiving placebo or simvastatin (Figure 3 and Table 4).

FBF responses to increasing doses of the vasoconstrictor NE were significantly blunted after LPS in the placebo group (Figure 4; \(P<0.05\) versus before LPS). Again, simvastatin prevented hyporeactivity to NE after LPS (Figure 4; \(P<0.05\) versus placebo).

**Discussion**

This study demonstrates that acute inflammation-induced forearm vascular dysfunction can be prevented by short-term pretreatment with high therapeutic doses of simvastatin in an experimental human model. Impairment of endothelium-dependent vasodilation is present in inflammation and was demonstrated in vitro\(^{23,24}\) and in resistance arteries of healthy humans challenged by different inflammatory stimuli.\(^{13,25}\) Consistent with previous studies, LPS impaired the FBF response to ACh and NE, with preservation of the dilation to the vascular smooth muscle relaxant NTG. This impairment of vascular function was prevented by pretreatment with simvastatin. In the present study, a significant decrease from high normal total cholesterol and LDL cholesterol levels was observed after simvastatin treatment. Although there was no significant difference in vascular function between the simvastatin and placebo groups after 4 days, this does not exclude a lipid-dependent effect of simvastatin in the present experiments. However, the present study cannot directly distinguish between lipid-dependent and -independent mechanisms of simvastatin, and thus, additional effects of simvastatin beyond lipid lowering may account for the preserved vascular function after LPS.

**TABLE 4. Absolute Forearm Blood Flow Values (mL·min\(^{-1}·100\ mL^{-1}\)) 4 Hours After LPS Administration in Subjects Receiving Placebo or Simvastatin**

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Placebo</th>
<th>Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 nmol/min</td>
<td>7.7±0.9</td>
<td>8.2±1.3</td>
</tr>
<tr>
<td>50 nmol/min</td>
<td>9.2±1.3*</td>
<td>13.0±2.0</td>
</tr>
<tr>
<td>100 nmol/min</td>
<td>9.6±1.2*</td>
<td>15.4±1.8</td>
</tr>
<tr>
<td>GTN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 nmol/min</td>
<td>7.6±0.8</td>
<td>8.0±1.1</td>
</tr>
<tr>
<td>8 nmol/min</td>
<td>11.9±1.4</td>
<td>12.1±1.5</td>
</tr>
<tr>
<td>16 nmol/min</td>
<td>13.7±1.6</td>
<td>13.8±1.8</td>
</tr>
</tbody>
</table>

Data are mean±SEM; \(n=10\) per group

\(^*P<0.05\) vs simvastatin (t test).
In previous studies, we have demonstrated that increased oxidative stress plays an important role in vascular dysfunction during inflammation. In those studies, LPS-induced hyporeactivity to ACh and to different vasoconstrictors could be reversed by intra-arterial infusion of high doses of the antioxidant vitamin C. Despite the lack of a direct antioxidative effect, statins may inhibit key proteins involved in generation of reactive oxygen species. Experimental studies revealed that statins inhibit production of superoxide via the NADPH oxidase system in monocytes, neutrophils, and endothelial cells, because activation of NADPH oxidase is dependent on an isoprenoid group, ie, geranyl geraniol, which derives from HMG-CoA and is thus inhibited by statins. In parallel to these in vitro observations, De Caterina and coworkers found a significant reduction of F2-isoprostane, a product of the attack of reactive oxygen species on esterified arachidonic acid, in patients with hypercholesterolemia who were receiving simvastatin. Statin therapy also lowers systemic levels of protein-bound nitrotyrosine, a marker for formation of peroxynitrite, ie, the end product of the reaction of superoxide with nitric oxide. A recent in vitro study shows that overproduction of superoxide anions can be prevented by simvastatin in monocytes from septic patients. Decreased systemic oxidative stress in response to LPS by simvastatin was evidenced by changes in spontaneous and FMLP-activated oxidative burst of neutrophils. Assessment of neutrophil oxidative burst by a dihydroindolcarbonyl flow cytometric assay in whole blood is considered a sensitive technique to estimate oxidative stress in vivo. In addition to antioxidative capacities, it has been shown that statins exert anti-inflammatory effects at high therapeutic or supratherapeutic concentrations. First, statins interfere with ligand receptor interaction in the inflammation cascade. Lovastatin, for instance, reduces the surface expression of CD11b on monocytes and thus reduces their adhesiveness to the vascular endothelium. It was also found that statins inhibit leukocyte function antigen-1, which is involved in leukocyte extravasation to sites of inflammation. Furthermore, the acute phase response, which includes a wide variety of mediators such as C-reactive protein, IL-6, TNF-α, and IL-1β, can be influenced by statins. In the present study, levels of IL-6, TNF-α, and IL-1β increased significantly during inflammation. In simvastatin-treated subjects, however, the increase in TNF-α levels was significantly reduced, which argues for an anti-inflammatory activity of simvastatin. IL-6 and IL-1β levels only tended to be nonsignificantly lower in the simvastatin group. This is probably due to the high interindividual variability in IL-6 levels, which was also observed after LPS administration in a previous study. There is a debate regarding the IL-6 response to statin therapy in the literature. Although some report a decrease, others report little or no effect on IL-6 levels. Finally, statins can also increase expression of the endothelium-derived nitric oxide synthe. Controversial reports exist about expression of the inducible nitric oxide synthase isoform (iNOS) and statins. No change was reported after simvastatin, whereas other studies demonstrated an inhibitory effect of atorvastatin. However, the role of iNOS in the human LPS model is probably very limited, because our previous trial failed to demonstrate increased iNOS mRNA and protein expression after LPS. The measurement of NOx levels in the present study was an attempt to detect changes in NO metabolism. Although no changes were observed in NOx levels, the results of these measurements are limited, because nitrate/nitrite intake, which is known to influence NOx levels, was not standardized during the study period.

In addition to prevention of endothelial dysfunction during inflammation, the response to NE was also preserved by simvastatin pretreatment. Interestingly, a recent review reported a significant reduction in both overall and attributable mortality among patients with bacteremia who received statins compared with controls. However, clinical diagnosis and concomitant diseases were slightly different in patients taking statin therapy. Nevertheless, given the important role of vasodilation and vasoconstrictor hyporeactivity in the development of septic organ dysfunction, it is possible that preserved vascular responsiveness during inflammation by statins may have contributed to improved outcome. This would be compatible with 2 recently published animal studies in which sepsis-related mortality was significantly reduced by simvastatin.

Although we did not compare simvastatin with other statins in the present experiments, data from other studies suggest that antioxidative and anti-inflammatory properties of statins may vary. Thus, additional studies in humans are needed to test whether these effects of statins represent a class effect or are specific for particular statins.

In conclusion, the present study demonstrates potent vasoprotective properties of high-dose simvastatin during acute inflammation. Whether this effect can also be seen in states of chronic inflammation remains to be elucidated.

Acknowledgments

We are grateful for the assistance and administrative work of Carola Fuchs, RN, Lana Nezic, MD, and the laboratory expertise of Sonja Kapiotis, MTA.

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Circulation. 2004;110:3349-3354; originally published online November 1, 2004; doi: 10.1161/01.CIR.0000147774.90396.ED
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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