Simvastatin Blunts Endotoxin-Induced Tissue Factor In Vivo

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Background—Beyond lipid lowering, various antiinflammatory properties have been ascribed to statins. Moreover, in vitro studies have suggested the presence of anticoagulant effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, as lipopolysaccharide (LPS)-induced monocyte tissue factor (TF) was suppressed. In this study, we examined the role of statins in experimental endotoxia on inflammatory and procoagulant responses in vivo.

Methods and Results—In this double-blind, placebo-controlled, parallel-group study, 20 healthy, male subjects were randomized to receive either simvastatin (80 mg/d) or placebo for 4 days before intravenous administration of LPS (20 IU/kg IV). Plasma high-sensitive C-reactive protein (hsCRP), monocyte chemoattractant protein (MCP-1), sCD40L, sCD40, and prothrombin fragment F1+2 (F1.2) were determined by ELISAs at baseline and at 4 and 8 hours after LPS administration. Monocyte TF expression and monocyte-platelet aggregates were measured by whole-blood flow cytometry over the same time course. The increases in hsCRP and MCP-1, both known inducers of TF, were significantly suppressed by statin treatment after LPS challenge. Statin premedication blunted the increase of monocyte TF expression in response to LPS. In parallel, endotoxin-induced formation of F1.2 was significantly reduced by simvastatin after 4 and 8 hours. LPS infusion affected neither the formation and activation of monocyte-platelet aggregates nor plasma levels of sCD40 and sCD40L.

Conclusions—Simvastatin suppresses the inflammatory response to endotoxin and blunts monocyte TF expression but does not affect platelet activation. (Circulation. 2005;111:1841-1846.)

Key Words: statins ■ inflammation ■ thrombosis

In large clinical trials, statin therapy has been shown to significantly reduce cardiovascular morbidity and mortality.1-3 In addition to lipid lowering, statins are postulated to exhibit pleiotropic, nonlipid properties such as improvement of endothelial function, plaque stabilization, and the reduction of oxidative stress in vascular inflammation.4 The antiinflammatory effect of statins appears to be mediated via interference with the synthesis of isoprenoid intermediates (mevalonate metabolites)5 and to limit nuclear factor-κB (NF-κB)-dependent transcriptional regulation in response to inflammatory stimuli.5,6 This mechanism may contribute to the previously reported clinical benefit of statin therapy in patients with sepsis.7,8 Beyond the established antiinflammatory effect,4 statins may interfere with coagulation activation, as proposed by studies that have demonstrated suppression of lipopolysaccharide (LPS)-induced monocyte tissue factor (TF) in vitro.9

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In the present study, we sought to investigate the role of statins in the initial phase of systemic inflammation and coagulation activation in experimental endotoxia by LPS injection into human volunteers. We hypothesized that pretreatment with statins might block LPS-induced monocyte TF expression and thrombin generation in vivo. Because platelets have been shown to enhance monocyte TF expression in LPS-stimulated whole blood in vitro,10 we further examined the effect of simvastatin on LPS-induced monocyte-platelet aggregate (MPA) formation in vivo. In addition, we determined levels of the proinflammatory markers high-sensitive C-reactive protein (hsCRP) and monocyte chemoattractant protein (MCP)-1 as well as the CD40/CD40L pathway after human LPS challenge.

Methods

Study Design and Subjects

Blood samples were collected from subjects who participated in a study that investigated vascular reactivity after LPS and simvastatin pretreatment.11 This study protocol was approved by the Ethics Committee of the Medical University of Vienna and complies with...
the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines. Written, informed consent was obtained from all study participants.

**Study Protocol**

The protocol of this study has been described in detail elsewhere. In brief, the study was designed as a randomized, double-blind, placebo-controlled trial in 2 parallel groups of 20 healthy, male volunteers (n=10 per group) between 20 and 40 years old. Participants were nonsmokers and had no history or signs of cardiovascular risk factors. Exclusion criteria consisted of regular or recent intake of any medication, including nonprescription drugs. Subjects were randomly assigned to receive 80 mg simvastatin or placebo as a single oral dose for 3 days. On the fourth day, subjects were admitted to the ward in the morning. After intake of the last study medication, volunteers received a bolus of LPS (20 IU/kg body weight IV, dose corresponding to 2 ng/kg; National Reference Endotoxin Escherichia coli, USP). Approximately 9 to 10 hours after LPS infusion, subjects were discharged in good health.

**Blood Sampling and Immunoassays**

Plasma samples were prepared immediately by centrifugation (2000 g at 4°C for 10 minutes) of peripheral venous blood that had been collected into evacuated tubes (Vacutainer, Becton Dickinson) containing 0.129 mmol/L sodium citrate as the anticoagulant at baseline (days 1 and 4) as well as at 4 and 8 hours after LPS infusion. Aliquots were stored at −80°C before analysis.

Commercially available ELISAs were used for measurement of prothrombin fragment FI+2 (Fl1.2; Enzygnost Fl1.2 micro, Dade Behring), MCP-1 (human MCP-1 ELISA, Bender MedSystems), sCD40L (human sCD40L ELISA, Becton MedSystems), sCD40 (human sCD40 ELISA, Bender MedSystems), and high-sensitive C-reactive protein (hsCRP, Imucine CRP ELISA, American Diagnostica) according to the manufacturer’s instructions.

**Cell Count and Flow Cytometry**

Generally, differential blood counts were determined by using a cell counter (Sysmex); however, because monocyte levels have been shown to be falsly high with this method in LPS-treated volunteers, monocyte counts were calculated from scatter histograms obtained by flow cytometry.

Whole-blood flow cytometry was performed as previously described with little modification. In brief, 100 μL of citrate-anticoagulated whole blood was stained with saturating concentrations of the following fluorochrome-conjugated monoclonal antibodies (mAbs): phycoerythrin (PE)-labeled mAb for human TF (Becton Dickinson), PE-Cy5–labeled mAb for monocyte CD14 (endotoxin receptor), PE-labeled mAb for the platelet activation marker CD62P (P-selectin), PE-labeled mAb for CD40L (CD40 ligand; all antibodies from Instrumentation Laboratories), APC-labeled mAb for the constitutive platelet marker CD42b (glycoprotein Ibα of von Willebrand factor receptor complex; Becton Dickinson), and corresponding isotype controls (Becton Dickinson and Instrumentation Laboratories). After 10 minutes of preincubation with antibodies in the dark at room temperature, the samples were fixed and erythroblyzed with Optilyse B (Instrumentation Laboratories). Flow cytometry was performed on a FACScalibur (Becton Dickinson). Acquisition was stopped when 3000 CD14+ events were acquired.

**Statistical Analysis**

Values are given as mean±SEM. Baseline comparisons between the simvastatin and placebo groups were made with t tests for independent samples. The 4-day statin effect compared with baseline was analyzed by a paired Student t test. General linear model repeated-measures ANOVA was used to test the response to LPS within the group and to test differences in the response to LPS between statin therapy and placebo over time. Significance was defined as P<0.05. All analyses were calculated with the use of SPSS software package 11.0 for Windows.

**TABLE 1. Clinical Characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Statin (n=10)</th>
<th>Placebo (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>28±2</td>
<td>26±1</td>
<td>NS</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76±3</td>
<td>77±5</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.8±1.0</td>
<td>23.4±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>125±3</td>
<td>127±6</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>69±3</td>
<td>68±4</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.4±0.3</td>
<td>4.5±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3±0.1</td>
<td>1.3±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.6±0.3</td>
<td>2.7±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.2±0.2</td>
<td>1.0±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

**Results**

Clinical characteristics and baseline values of blood counts, MPAs, monocyte TF expression, and circulating procoagulant and inflammatory parameters are listed in Tables 1 and 2, showing no significant difference between the statin group and controls. Except for a significant reduction of total cholesterol in the statin group (day 1, 4.4±0.3 mmol/L versus day 4, 3.6±0.3 mmol/L; P<0.05), no change in measured parameters was observed after 3 days of statin therapy. In the placebo group, baseline values of all parameters were comparable between days 1 and 4, including total cholesterol measurements (day 1, 4.5±0.3 mmol/L versus day 4, 4.5±0.3 mmol/L; P=NS).

**Changes in Differential Leukocyte Count and Platelet Count**

Four hours after LPS infusion, the number of leukocytes was nearly doubled compared with baseline (1.9±0.2-fold increase) and was still 1.7±0.2-fold increased after 8 hours (P<0.05). The monocyte count significantly dropped by 82±2% at 4 hours after LPS infusion but returned to 72±3%.
of the baseline value 8 hours after LPS administration ($P<0.05$). No difference was observed for the degree of monocytopenia between the statin and placebo groups. Because of the decreased monocyte counts, flow-cytometric acquisition of CD14$^+$ events was insufficient ($<3000$ events) in 5 samples at the 4-hour time point (3 in the statin group). These results were excluded from calculations. Platelet counts dropped by a maximum of 17±5% after 4 hours of LPS challenge and were still decreased by 8±4% at 8 hours thereafter ($P<0.05$).

**Statin Treatment Blunts LPS-Induced Procoagulant and Inflammatory Responses**

LPS challenge increased monocyte TF expression, measured as the percentage of TF$^+$ monocytes (CD14$^+/TF^+$/100), after 4 and 8 hours, which was completely blunted by statin pretreatment (4 hours, 1.7±0.2-fold versus 0.9±0.1-fold increase; 8 hours, 2.0±0.3-fold versus 1.0±0.2-fold increase; $P<0.05$; Figure 1A). In addition to the proportion of TF$^+$ monocytes, TF mean fluorescence intensity, which provides a marker of the epitope density of TF molecules on monocytes, was lower in the statin treatment group compared with the placebo group after LPS infusion (Table 3).

**TABLE 3. TF Expression as a Function of Time After LPS Challenge**

<table>
<thead>
<tr>
<th>Timecourse</th>
<th>%CD14$^+$/TF$^+$</th>
<th>TF MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statin</td>
<td>Placebo</td>
</tr>
<tr>
<td>−72 h</td>
<td>14.9±2.3</td>
<td>16.7±2.3</td>
</tr>
<tr>
<td>0 h</td>
<td>16.4±3.1</td>
<td>16.8±1.5</td>
</tr>
<tr>
<td>4 h</td>
<td>13.2±2.1</td>
<td>27.9±2.3</td>
</tr>
<tr>
<td>8 h</td>
<td>16.0±2.3</td>
<td>32.7±4.0</td>
</tr>
</tbody>
</table>

*Data are expressed as mean±SEM. Probability values represent general linear model repeated-measures ANOVA comparing statin treatment with placebo over time.*

In parallel, endotoxin-induced formation of F1.2 was significantly decreased by simvastatin in timecourse (4 hours, $8.7±1.3$-fold versus $4.7±0.7$-fold increase; 8 hours, $6.4±0.7$-fold versus $3.9±0.7$-fold increase; $P<0.05$; Figure 1B) hours. MCP-1 secretion transiently increased 4 hours after LPS stimulation but rapidly declined thereafter; at both time points, statin therapy suppressed MCP-1 plasma levels (4 hours, 80.0±8.3-fold versus 57.3±5.2-fold increase; 8 hours, 5.2±1.2-fold versus 2.5±0.3-fold increase; $P<0.05$; Figure 1C). The increase in hsCRP was also significantly diminished by treatment with simvastatin (4 hours, 1.5±0.2-fold versus 1.0±0.2-fold increase; 8 hours, 6.6±0.6-fold versus 3.5±0.8-fold increase; $P<0.05$; Figure 1D).

**LPS Challenge Does Not Affect Platelet Activation**

MPA formation (CD14$^+$/CD42b$^+$) and the proportion of activated platelets (characterized by P-selectin [CD14$^+$/CD62P$^+$] and CD40L [CD14$^+$/CD40L$^+$] expression associated with monocytes did not differ in time course (Figure 2A through 2C). Soluble CD40L and CD40 measured in plasma were also unaltered by LPS stimulation (Figure 3A and 3B).

**Discussion**

Several clinical trials have established a role for statin therapy in the primary and secondary prevention of atherosclerosis.$^{1–3}$ Beyond lipid lowering, antiinflammatory effects have been attributed to statin treatment,$^{14,15}$ which appears to be exceedingly beneficial in patients with elevated parameters of inflammation such as hsCRP and sCD40L.$^{16–18}$ In addition, statin therapy has been associated with a decreased rate of severe sepsis and reduced mortality in patients with acute bacterial infection.$^{7,8}$ In septicemia, invading microorganisms as well as defense mechanisms lead to systemic activation of the immune system and the coagulation cascade,$^{19}$ both potential targets of statin therapy.

Here we show for the first time potent inhibition of LPS-induced inflammatory and procoagulant responses by...
We found that short-term pretreatment of human volunteers with simvastatin significantly reduced plasma levels of hsCRP and MCP-1 as well as monocyte TF expression and TF-dependent F1.2 formation after intravenous injection of LPS, an established model of early systemic coagulation activation during endotoxemia.

Our finding is in line with previous studies that reported a reduction in hsCRP after long-term statin treatment in vivo, however, we showed early suppression of the LPS-induced inflammatory response, as demonstrated by diminished hsCRP, after only 4 days of pretreatment with high-dose simvastatin. Furthermore, we confirmed the antiinflammatory effect of statins in human endotoxemia by showing inhibition of MCP-1 induction. This is in line with a previous in vitro study that reported dose-dependent suppression of LPS-induced MCP-1 in peripheral blood mononuclear cells and a reduction of MCP-1 in an air-pouch model of local inflammation in mice.21 The anticoagulant property of statins has been proposed before by the authors of several in vitro studies, which showed diminished LPS-induced monocyte TF and TF-dependent thrombin generation.9,22,23

On ligation of CD14 on the cell surface of monocytes, LPS stimulates the induction of several inflammatory genes by activating intracellular signaling pathways, including the IκB kinase–NF-κB pathway.24 Recently, statins were shown to prevent activation of NF-κB by...
upregulation of the NF-κB inhibitory protein IkB. This mechanism was primarily dependent on the suppression of mevalonate-derived products. Because the expression of MCP-1 and monocyte TF is dependent on NF-κB-regulated transcription, this may be a major mechanism for the observed antiinflammatory/anticoagulant effect of statins in endotoxemia in vivo.

Platelets (and thus, platelet-leukocyte cross-talk) have been demonstrated to enhance monocyte TF expression after LPS-stimulation of whole blood in vitro. Thus, we evaluated the role of LPS challenge on MPA formation in vivo, previously shown to be a more sensitive marker of platelet activation than platelet P-selectin expression. Surprisingly, despite documented monocyte activation and TF-dependent thrombin generation, MPA formation did not increase in response to LPS in our study and was not influenced by statin pretreatment. Platelet activation in MPAs, as measured by P-selectin and CD40L expression, did not change in time course after LPS challenge. The minor role of platelet activation in this model of human endotoxemia is supported by another study showing no effect of antiplatelet therapy with aspirin on monocyte TF expression. Additionally, we confirmed the lack of significant platelet activation by measuring sCD40L, usually shed into plasma by activated platelets, which did not increase after administration of LPS. This may be explained by the previously described downregulation of protease-activated receptor 1 on platelets with decreased platelet responsiveness to thrombin stimulation during systemic inflammation.

Short-term statin treatment had no effect on plasma levels of sCD40L and sCD40. This finding is in contrast to the previously described decrease of sCD40L and sCD40 observed weeks after initiation of statin therapy; however, statin treatment may rather play a role in patients with hypercholesterolemia, with known upregulation of sCD40 and sCD40L, than in apparently healthy, normolipemic subjects.

Here, we studied only the effect of 4 days of statin pretreatment on inflammatory and procoagulant responses to LPS-induced sepsis for 2 reasons: first, to keep the study and placebo groups homogenous and second, as an in vivo proof of concept, as suggested by previous in vitro studies. Prolonged statin medication may have an even more pronounced effect on the described parameters, as clinical studies have shown improved outcomes in patients with sepsis taking statins for at least 1 month before bacterial infection.

In summary, our results suggest that statins profoundly reduce the inflammatory as well as the associated procoagulant response in this human model of sepsis by reduction of hsCRP, MCP-1, monocyte TF, and TF-dependent thrombin generation. To evaluate the role of statins as a primary prevention approach to sepsis, larger randomized, clinical trials are warranted.

References


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