HMG-CoA Reductase Inhibitor Simvastatin Profoundly Improves Survival in a Murine Model of Sepsis

Marc W. Merx, MD; Elisa A. Liehn, MD; Uwe Janssens, MD; Rudolf Lütticken, MD; Jürgen Schrader, MD; Peter Hanrath, MD; Christian Weber, MD

Background—HMG-CoA reductase inhibitors, such as simvastatin, have been shown to exhibit pronounced immunomodulatory effects independent of lipid lowering but to date have not been used to treat severe inflammatory disease such as sepsis. We thus approached the question of whether treatment with simvastatin might improve cardiovascular function and survival in sepsis.

Methods and Results—Mice treated with simvastatin and rendered septic by cecal ligation and perforation (CLP) show a mean survival time close to 4 times the value found in untreated mice. This dramatic improvement is based on a complete preservation of cardiac function and hemodynamic status, which are severely impaired in untreated CLP mice [e.g., 20 hours after CLP, cardiac output declined from 1.24±0.09 to 0.87±0.11 mL · min⁻¹ · g⁻¹ in untreated mice (P<0.005; n=12), while remaining unaltered (1.21±0.08 mL · min⁻¹ · g⁻¹ at baseline and 1.15±0.1 mL · min⁻¹ · g⁻¹ 20 hours after CLP, P=NS, n=12) in CLP mice treated with simvastatin]. Untreated CLP mice remained refractory to β-stimulation, whereas the responsiveness to dobutamine was restored by treatment with simvastatin. Susceptibility of coronary flow to endothelial nitric oxide synthase (eNOS) stimulation by bradykinin was close to 3 times as pronounced in untreated CLP mice as in untreated sham-operated mice, indicating a high level of eNOS activation secondary to sepsis. In addition, treatment with simvastatin reversed inflammatory alterations in CLP mice, namely, increased monocyte adhesion to endothelium.

Conclusions—Simvastatin, which is well established in the treatment of lipid disorders and coronary artery disease, might have the additional potential of being an effective agent in sepsis treatment. (Circulation. 2004;109:2560-2565.)

Key Words: sepsis ■ inflammation ■ hemodynamics ■ leukocytes ■ statins

Despite extensive research invested in the field over more than 2 decades (for review, see Hotchkiss and Karl1 and Riedemann et al), sepsis remains the leading cause of death among patients treated in intensive care units, with mortality rates ranging between 30% and 70%.2 Sepsis is generally viewed as a disease aggravated by the inappropriate and inefficient immune response encountered in the affected individual. Thus, basic research and clinical trials have focused on agents capable of blocking steps within the inflammatory cascade.

Corticosteroids,3,4 activated protein C,5 tumor necrosis factor (TNF) antagonists,6 interleukin-1 receptor antagonists,7 anti-endotoxin antibodies,8 and ibuprofen9 have all been evaluated in a clinical setting, with improved outcome demonstrated recently for activated protein C.5 Promising studies in animal models of sepsis have focused on high-mobility group B1 protein,10 complement C5a, its receptor C5aR, and macrophage migration inhibitory factor.11 HMG-CoA reductase inhibitors (statins) such as simvastatin have been shown to exhibit important immunomodulatory effects independent of lipid lowering.12 These pleiotropic effects have been demonstrated to include anti-inflammatory actions,13 improvement of endothelial and microvascular function, and modulation of endothelial nitric oxide synthase (eNOS).14 However, statins have thus far not been used to treat severe inflammatory states such as sepsis. We thus approached the question of whether treatment with simvastatin might improve cardiovascular function and survival in a murine model of sepsis.

Methods

Animals

Male C57 mice were treated twice by intraperitoneal injection (IP; 18±2 hours and 3±1 hours before cecal ligation and perforation [CLP] or sham operation) of simvastatin (Merck Biosciences; 0.2 μg/g body weight [BW]; injected volume, 0.02 mL/g BW) or placebo (carrier only, 0.02 mL/g BW) and rendered septic by CLP with respective sham-operated animals functioning as controls. Simvastatin was dissolved in EtOH at a concentration of 10 mg/mL and diluted with NaCl 0.9% at a ratio of 1:1000 to yield a final concentration of 10 μg simvastatin/mL carrier. The NaCl 0.9%-

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Sepsis Induction and RR Measurements

Anesthesia was induced by IP administration of ketamine (60 \( \mu g/g \) BW) and xylazine (10 \( \mu g/g \) BW). Through a 1-cm abdominal midline incision, the cecum was ligated below the ileocecal valve with careful attention to avoiding obstruction of the ileum or colon. The cecum was subjected to a single "through-and-through" perforation (20-gauge needle). The abdominal incision was closed in layers. Sham-operated mice underwent the same procedure except for ligation and perforation of the cecum. Food and water were provided ad libitum. Pain medication (tramadol 20 \( \mu g/g \) BW) and volume support (NaCl 0.9%, 0.05 mL/g BW) were applied subcutaneously immediately after the induction of sepsis and every 6 hours thereafter. For RR measurements, anesthesia was induced as above 20±2 hours after surgery. The carotid artery was cannulated, and pressure was recorded at baseline and after IP injection of dobutamine (1.5 \( \mu g/g \) BW) and ethylthiourea (ETU) (1 \( \mu mol/g=185.1 \mu g/g \) BW), respectively.

Langendorff Setup and Protocol

Hearts were isolated 20±2 hours after surgery, and retrograde perfusion was performed essentially as described previously, using a commercially available isolated heart apparatus (Hugo Sachs Elektronik). Perfusion pressure, perfusate oxygen concentration, aortic flow, left ventricular pressure, and heart rate were measured continuously (PC with A/D converter (2000 Hz) and dedicated software; EMKA Technologies). Derivative parameters (dP/dt_{max}, dP/dt_{min}, coronary flow, tau, relaxation half-time, and time to peak pressure) were displayed in real time and recorded. Hearts were stimulated at 600 bpm. Bradykinin and adenosine were applied at concentrations of 5 \( \mu mol \) and 1 \( \mu mol \), respectively.

Echocardiography

Conscious mice (n=12 per group, 28 to 38 g) were examined by echocardiography 20±2 hours after surgery as described previously. Using a 15-MHz linear transducer connected to a Sonos 5500 (Philips Medical Systems; frequency fusion, 5; interrogation depth, 2 cm), the heart was imaged in 2D mode in the parasternal long-axis view, and 2D guided M-mode images were obtained at the aortic root for offline aortic diameter measurements. Aortic flow velocity was measured with pulsed-wave Doppler with the reference volume being placed just above the aortic root and careful angle adjustment strictly parallel to the ascending aorta.

Aortic flow velocity time integral (VTI) and aortic root dimension (AoD) were obtained from the respective frozen images as above. Cardiac output (CO) was calculated from the following equations: \( CSA=\frac{(AoD)^2\sqrt{\pi}}{2} \), SV=\( CSA\times VTI \), and CO=\( SV\times HR \), where \( CSA \) is aortic cross-sectional area, \( SV \) is stroke volume, and \( HR \) is heart rate. The same procedure was followed after dobutamine (1.5 \( \mu g/g \) BW) application. Images were obtained within 7 and 11 minutes after intraperitoneal injection, a time frame shown to represent the plateau pharmacological effect in preliminary experiments.

Cell Isolation, Cell Culture, and Shear Flow

Blood samples were taken from mice (n=8 per group) by cardiac puncture 24 hours after operation. Mononuclear cells were isolated, and cell suspensions were washed and resuspended (5×10^8) in HHMC (HBSS, 10 mmol/L HEPES, 1 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}, 0.5% BSA). Viability was >97%.

WEHI-274.1 (mouse monocytes, ATCC) were cultured in DMEM (+2 mmol/L glutamine+0.05 mmol/L 2 mercaptoethanol+10% FBS). Cells were incubated with 1 \( \mu mol \) simvastatin, 1 \( \mu mol \) pravastatin (Merck Biosciences), or 1 \( \mu mol \) simvastatin and 100 \( \mu mol \) mevalonic acid for 30 minutes or 24 hours or left untreated, washed, and resuspended (1×10^6) in HHMC.

SVECs (SV40-immortalized murine endothelial cells, kindly provided by Dr H. Hengel, Berlin) were cultured in DMEM (+5% FKS), grown to confluence in 35-mm Petri dishes, stimulated with TNF-\( \alpha \) (200 U/mL, Sigma), and incubated with 1 \( \mu mol \) simvastatin or with 1 \( \mu mol \) simvastatin and 100 \( \mu mol \) mevalonic acid for 30 minutes or 24 hours or were left untreated. Cell dishes were mounted on the stage of an Olympus IX50 microscope as described previously. Mononuclear cells or WEHI-274.1 monocytes were perfused into the flow chamber at a rate of 1 dyne/cm^2 for 7 minutes, and adherent cells were counted.

Statistical Analysis

Mean values with SD are reported. Differences in repeated measurements were analyzed by multivariate ANOVA followed by Bonferroni’s post hoc test. Otherwise, a Student’s t test was applied (SPSS 10.0).

Results

Survival After CLP Is Profoundly Prolonged in Simvastatin-Treated Mice

Mice were treated with simvastatin or placebo. Sepsis was induced via CLP, with sham-operated animals serving as controls. No deaths occurred in the sham-operated animals whether or not they had been treated with simvastatin. The survival curves for mice in which CLP was performed (Figure 1) clearly delineate the benefit sustained from simvastatin treatment, with median survival time extended to 108 hours from 28 hours in untreated mice (n=10, P<0.005).

Blood cultures obtained at 20 hours after CLP confirmed bacteremia in both treated and untreated mice, whereas no pathogens could be cultured out of blood drawn from sham-operated mice. The pathogens identified were Enterococcus cloacae, Escherichia coli, Proteus mirabilis, and Alcaligenes faecalis. These species were found both in simvastatin-treated and untreated CLP mice.

Cardiac Function and Hemodynamics Are Completely Preserved by Simvastatin Treatment

To address possible changes in cardiac output secondary to the sepsis induced, we studied conscious mice by echocardiography.
Susceptibility to eNOS Stimulation and Coronary Flow Reserve

In agreement with the findings from conscious and anesthetized mice, simvastatin treatment completely preserved contractility of isolated hearts, which was impaired by 28% in untreated CLP mice compared with sham-operated animals ($P<0.001$, $n=8$; Table). Similarly, left ventricular developed pressure was also decreased solely in untreated CLP mice (Table), as were other parameters of contraction and relaxation (eg, time to peak pressure, relaxation half-time) and oxygen consumption (data not shown).

Baseline coronary flow was increased by 33% in untreated CLP mice compared with CLP mice treated with simvastatin (Figure 3). No significant difference was detected in basal coronary flow between the latter and sham-operated animals whether or not they were treated with simvastatin. Susceptibility to eNOS stimulation by bradykinin was close to 3 times as pronounced in untreated CLP mice as in untreated sham-

<table>
<thead>
<tr>
<th>Contractile Function of Isolated Hearts</th>
<th>CLP</th>
<th>CLP + Simvastatin</th>
<th>Sham + Simvastatin</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>% dP/dtmax mm Hg/s</td>
<td>3687±933*</td>
<td>4845±665</td>
<td>4995±743</td>
<td>4723±637</td>
</tr>
<tr>
<td>LVDP, mm Hg</td>
<td>86.7±14.6†</td>
<td>111.7±8.2</td>
<td>112.4±7.6</td>
<td>110.8±7.3</td>
</tr>
</tbody>
</table>

LVDP indicates left ventricular developed pressure. Values are mean±SD. Untreated CLP mice showed significant contractile impairment ($P<0.01$ and $\ddagger P<0.01$; sample size, $n=8$ for all groups), whereas simvastatin-treated CLP mice remained at par with sham-operated mice.
To investigate the effects of sepsis and simvastatin treatment on leukocyte-endothelial interaction, mononuclear cells were isolated from all groups of mice (CLP, CLP+simvstatin, sham, and sham+simvstatin) and subjected to adhesion assays on cytokine-stimulated murine endothelial cells under physiological flow conditions. The adhesion of monocytes isolated from CLP mice was significantly increased compared with that of sham-operated mice (Figure 4). Cells from simvastatin-treated animals displayed a reduced adhesion compared with cells isolated from untreated animals. No difference was observed between the adhesion of cells from CLP mice treated with simvastatin and sham-operated mice (Figure 4).

The effect of simvastatin was also evaluated in vitro by treatment of WEHI-274.1 cells and stimulated SVECs with simvastatin for 30 minutes or 24 hours. The number of adherent cells was reduced in all cases (Figure 5). Because the effect was evident after 30 minutes and was not reversible after addition of mevalonic acid, a short-term exposure appears to be sufficient for inhibition of adhesion, hinting at a direct interference with lymphocyte function–associated antigen (LFA-1) activity. To substantiate that the effect of simvastatin was related to an interference with LFA-1 activity, monocytes were pretreated with pravastatin (known to not interact with LFA-1) for 30 minutes before adhesion assays. Notably, the arrest of monocytes was not altered by pravastatin (115.5±6.0 cells/mm², n=3, P=NS versus control), suggesting that the effect was dependent on interference of statins with LFA-1.

After 24 hours, the effect of simvastatin was almost completely reversible by coincubation with mevalonic acid.
only in monocytes but not in endothelial cells or when treating both cell types. This indicates that in monocytes, the interference of simvastatin with the mevalonic acid–dependent pathway (eg, inhibition of Rho GTPase membrane localization and activity) appears to be reversible after 24 hours, whereas the mevalonic acid–independent effects of simvastatin appear to prevail in endothelial cells (eg, activation of eNOS synthetase activity).

Discussion
To the best of our knowledge, the present study is the first to demonstrate that HMG-CoA reductase inhibitors significantly improve survival in sepsis. The dramatic improvement in survival observed here stems from the complete preservation of cardiac function and hemodynamic stability observed only in treated mice. As one of the underlying mechanisms, we demonstrate increased mononuclear cell adhesiveness in septic mice, an important contributor to sepsis pathophysiology, to be reversed by statin treatment.

We chose the CLP model, because it closely resembles the pathophysiology of human sepsis. We observed bacteremia as well as hemodynamic alterations typically found also in patients affected by sepsis, namely, a hyperdynamic (data not shown) followed by a hypodynamic physiological state. The latter is characterized by decreased cardiac output and low peripheral resistance. We demonstrate that in our model, impaired cardiac output is a consequence of the decreased contractility of septic hearts and that this acute septic cardiomyopathy is refractory to catecholamine stimulation, a fact paralleled in human pathophysiology and often presenting a formidable challenge during the treatment of septic patients. The reduced arterial blood pressure of septic mice, in conjunction with the limited rise in blood pressure observed after nonselective NOS inhibition, suggests that reduced peripheral vascular resistance is mediated by NO and other vasodilatory agents. Indeed, anaerobic metabolites such as lactic acid are frequently encountered in septic patients and result in pathologically low pH values, accounting for both vasodilation and catecholamine resistance. The pronounced cardiac dysfunction and hypodynamic circulation present severe manifestations of sepsis, contributing to the poor outcome and short survival time documented in the present septic mouse model. As survival time is a relatively crude parameter of treatment benefit, it is remarkable that we found a nearly 4-fold increase of median survival time in simvastatin-treated septic mice. Indeed, improvement by statin therapy was so robust that cardiac function and hemodynamic status remained completely unaffected.

On the vascular level, sepsis produces endothelial dysfunction and monocyte activation. We isolated peripheral blood mononuclear cells and demonstrated their adhesion to TNF-α–stimulated endothelial cells. TNF-α mimics sepsis by enhancing the endothelial expression of the adhesion molecules E-selectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule, and chemokines and cytokines, after activation of nuclear factor-κB and the extracellular signal–regulated kinase/mitogen-activated protein kinase cascade. As expected, we found the adhesion of monocytes isolated from animals rendered septic by CLP to be increased. However, the adhesion of monocytes isolated from simvastatin-treated septic animals was markedly attenuated.

Statins are well established for the treatment of hypercholesterolemia, with the clinical benefit commonly attributed to reduced cholesterol synthesis. The latter is achieved by inhibiting the conversion of HMG-CoA to mevalonate. Because mevalonate is the precursor not only of cholesterol but also of many other nonsteroidal isoprenoid products, HMG-CoA reductase inhibition might affect several other cellular functions. In this context, suppression of T-cell responses reduced expression of class II major histocompatibility complexes on antigen-presenting cells, and reduced chemokine synthesis in peripheral blood mononuclear cells has been demonstrated. In addition, it was recently reported by Weitz-Schmidt et al that several statins (including simvastatin) are capable of blocking the LFA-1–ICAM-1 interaction, providing a mevalonate and thus HMG-CoA reductase–independent pathway for anti-inflammatory and immunomodulatory statin actions. In accordance with these findings, in our in vitro experiments, even a very short incubation of monocytes with simvastatin but not pravastatin resulted in reduced adhesion of monocytes to endothelium, with the latter not being reversible after addition of mevalonic acid. This effect might be explained by direct passive interaction of simvastatin with the metal ion–dependent adhesion site of LFA-1 I-domain that induces structural changes within the L-site region. This explanation is further substantiated by the absence of any effect of pravastatin on monocyte adhesion (with pravastatin known to differ from simvastatin in that it does not interact with LFA-1). However, other mechanisms, such as decreased CD11b expression and reduced CD11b–dependent adhesion of monocytes on endothelium, also demonstrated for simvastatin, cannot be excluded.

In summary, simvastatin, being well established in the treatment of lipid disorders and coronary artery disease, might have the additional potential of being an effective agent in sepsis treatment. We believe that the unexpected and promising results presented here warrant further, more clinically oriented animal studies, including the investigation of treatment after sepsis induction. In addition, because of the widespread application of statins, retrospective studies analyzing septic patients coincidentally treated with statins could pave the way toward prospective studies.

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