Statin Treatment After Onset of Sepsis in a Murine Model Improves Survival

Marc W. Merx, MD*; Elisa A. Liehn, MD*; Jürgen Graf, MD; Annette van de Sandt; Maren Schaltenbrand; Jürgen Schrader, MD; Peter Hanrath, MD; Christian Weber, MD

Background—HMG-CoA-reductase inhibitors have been shown to exhibit pronounced immunomodulatory effects independent of lipid lowering. We have recently demonstrated that pretreatment with simvastatin profoundly improves survival in a cecal ligation and perforation (CLP) model of sepsis. Here, we studied whether treatment with simvastatin after onset of sepsis-induced hemodynamic alterations is beneficial and whether prolonged survival can also be achieved with other statins.

Methods and Results—Mice were rendered septic by CLP. At 6 hours after sepsis induction, when profound hemodynamic alterations were manifest, treatment with atorvastatin, fluvastatin, pravastatin, simvastatin, or placebo was initiated. Except for fluvastatin (27±2.3 hours), survival time was extended from 23±1.2 hours for placebo-treated mice to 37±3.6 hours for simvastatin-treated, to 40±4.2 hours for atorvastatin-treated, and to 39±3.9 hours for pravastatin-treated mice. This profound improvement is based on the preservation of cardiac function and hemodynamic status in statin-treated animals, both of which are severely impaired in untreated CLP mice. As underlying mechanisms, improved susceptibility to endothelial nitric oxide synthase stimulation and reduced endothelial adhesion of leukocytes could be demonstrated after statin treatment.

Conclusions—Well established in the treatment of lipid disorders and coronary artery disease, statins harbor the additional and novel potential of effective sepsis treatment. This benefit extends to several but not all statins tested. (Circulation. 2005;112:117-124.)

Key Words: hemodynamics □ inflammation □ leukocytes □ sepsis
namic stability observed only in treated mice. As an underlying mechanism, we demonstrated in septic mice that increased monocellular cell adhesiveness, an important contributor to sepsis pathophysiology, was reversed by statin treatment. In a clinical, prospective, observational cohort study, Almog et al added strong supportive evidence with reduced rate of severe sepsis in patients pretreated with a statin for ≥1 month before hospital admission for acute bacterial infection.

In the present study, we approached the question of whether treatment with simvastatin after the onset of homeodynamic alterations might improve cardiovascular function in sepsis. Because antiinflammatory properties are known to vary between individual statins, we compared the effect of simvastatin with respect to cardiac output (CO) and sepsis survival with several other statins. We investigated atorvastatin as a powerful repressor of major histocompatibility class II, pravastatin as a hydrophilic substance without LFA-1 inhibitory properties, and fluvastatin, which can induce cyclooxygenase-2. To elucidate underlying mechanisms, stimulation of endothelial nitric oxide synthase (eNOS) and endothelial adhesion of leukocytes were examined ex vivo and in vitro.

Methods

Animals

Male C57 mice were kept according to federal regulations. All studies were approved by the local ethics committee and the state animal welfare commission. All animals had ad libitum access to standard chow and water. Mice were kept under pathogen-free conditions (specific pathogen free) and under a 12-hour day/night cycle. All studies were performed at the same time of day to obviate circadian influences. Mice ranged in body weight from 24 to 36 g and in age from 12 and 18 weeks. Mice were divided into subgroups matched for age and body weight as outlined later.

Sepsis Induction

Sepsis was induced as described previously. In brief, anesthesia was induced by intraperitoneal administration of ketamine (60 µg/g body weight) and xylazine (10 µg/g body weight). The cucm was then subjected to a single “through and through” perforation with a 20-gauge needle. Sham-operated mice underwent the same procedure except for ligation and perforation of the cucm. Preoperatively and postoperatively, all mice had unlimited access to chow and water. In addition, analgesia (tramadol 20 µg/g body weight) was applied subcutaneously immediately after the induction of sepsis and every 6 hours thereafter. At the same time points, volume support (NaCl 0.9%, 0.05 mL/g body weight, prewarmed) was administered through subcutaneous injection.

Six and 12 hours after sepsis induction, age- and body weight-matched groups of 10 mice each were treated by intraperitoneal injection of atorvastatin, fluvastatin, pravastatin, simvastatin (0.2 µg/g body weight; injected volume, 0.02 mL/g body weight), or placebo (carrier only, 0.02 mL/g body weight). Statins were dissolved in EtOH at a concentration of 10 mg/mL and diluted with NaCl 0.9% in a ratio of 1:1000 to yield a final concentration of 10 µg statin per 1 mL carrier. The NaCl 0.9%–based carrier solution for the placebo group was prepared accordingly to include EtOH only (ie, without statin) at a concentration of 1:1000.

Blood Pressure Measurements

Anesthesia was induced as above in CLP simvastatin-treated, CLP placebo-treated, sham simvastatin-treated, and sham placebo-treated mice matched for age and body weight (10 per group) 20±2 hours after sepsis induction. The carotid artery was cannulated, and pressure was recorded at baseline and after intraperitoneal injection of dobutamine (1.5 µg/g body weight) and ethylthiourea (ETU; 1 µmol/g = 185.1 µg/g body weight).

Langendorff Setup and Protocol

Preparation of murine hearts explanted from CLP simvastatin-treated, CLP placebo-treated, sham simvastatin-treated, and sham placebo-treated mice matched for age and body weight (10 per group) was initiated 20±2 hours after sepsis induction, and retrograde perfusion at 100 mm Hg constant pressure with modified Krebs-Henseleit buffer was performed essentially as described, with a commercially available isolated heart apparatus (Hugo Sachs Elektronik). Perfusion pressure, perfusate oxygen concentration, aortic flow, left ventricular developed pressure (the difference between minimal diastolic pressure and maximal systolic pressure), and heart rate were measured continuously. Hearts were stimulated at 600 bpm. Bradykinin and adenosine were applied at a concentration of 5 and 1 µmol, respectively.

Echocardiography

Conscious CLP statin-treated (atorvastatin, simvastatin, pravastatin, or fluvastatin), CLP placebo-treated (10 per group), sham statin-treated (atorvastatin, pravastatin, or fluvastatin), and sham placebo-treated mice matched for age and body weight (6 per group) were examined by echocardiography 20±2 hours after sepsis induction as described previously. To avoid bradycardia, seen occasionally when echocardiography is performed in awake mice, mice were trained to tolerate the handling associated with echocardiographic examination on 3 to 4 separate occasions per day over a period of 3 days. After this training period, all mice remained calm during the examination. If image recording of sufficient quality could not be initiated within 2 minutes, the animal was allowed to rest, and images were obtained later to ensure minimal time span of animal handling.

Using a 15-MHz linear transducer connected to a Sonos 5500 (Phillips Medical Systems), we obtained 2D guided M-mode images at the aortic root for offline aortic diameter measurements. Aortic flow velocity was measured with pulsed-wave Doppler. Aortic flow velocity-time integral (VTI) and aortic root dimension (AoD) were obtained from the respective frozen images as above, and mean values from 3 to 6 heartbeats were used for further analysis. CO was calculated from the following equations: CSA = (AoD/2)²×π, SV = CSA×VTI, and CO = SV×HR, where CSA is aortic cross-sectional area, SV is stroke volume, and HR is heart rate.

The same procedure was followed for echocardiography involving dobutamine (1.5 µg/g body weight) 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, Culture, and Shear Flow

Blood samples were taken from weight- and age-matched mice treated by intraperitoneal injection of atorvastatin, fluvastatin, pravastatin, or simvastatin as described above (n=8 per group) by cardiac puncture 24±2 hours after sepsis induction. Mononuclear cells were isolated from mouse blood by gradient centrifugation with Lympholyte-Mammalian (Cedarlain). Cell suspensions were washed and resuspended (5×10⁶ cells) in HHMC (HBSS, 10 mmol/L HEPES, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.5% BSA). Viability was >97% as determined by trypan blue dye exclusion.

WEHI-274.1 (mouse monocytes, ATCC) were cultured in DMEM (+2 mmol/L glutamine +0.05 mmol/L l-mercaptoethanol +10% FBS) as described by ATCC. For the experiments, the cells were incubated with 1 µmol atorvastatin, fluvastatin, or pravastatin with or without the addition of 100 µmol mevalonic acid for 30 minutes and 24 hours or were left untreated, washed, and resuspended (1×10⁶ cells) in HHMC. 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 tumor necrosis factor (TNF)-α (200 U/mL; Sigma), and incubated with 1 μmol atorvastatin, fluvastatin, or pravastatin with or without 100 μmol mevalonic acid for 30 minutes or 24 hours or left untreated. The cells were washed, and the dishes were assembled as the lower wall in a parallel-wall flow chamber and mounted on the stage of an Olympus IX50 microscope.24,25 Mononuclear cells or WEHI-274.1 monocytes were perfused into the flow chamber at a rate of 1 dyne/cm² for 7 minutes. After 3 minutes, the adherent cells were counted in multiple high-power fields and recorded with a JVC 3CCD video camera.

The investigator responsible for cell counts in the above-mentioned assays was blinded to treatment regimen.

Statistical Analysis
Mean values with appropriate SEM are reported. All study groups analyzed were initially tested for normality with the Kolmogorov-Smirnov procedure with Lilliefors correction. By this assessment, all groups studied fulfilled the criteria of normal distribution.

Groups were then analyzed by ANOVA, followed by Dunnett-T3 post hoc test for comparisons between groups. For cell studies, Newman-Keuls multiple-comparison test was used as a post hoc test for comparisons between groups after the initially preformed ANOVA. A value of P<0.05 was taken to indicate statistical significance. All statistical analyses were calculated with SPSS 12.0 (SPSS Inc).

Results
Sepsis Survival Is Extended by Statin Treatment
Sepsis was induced through CLP, with sham-operated animals serving as controls. In preliminary experiments, we observed a hyperdynamic followed by a hypodynamic physiological state, similar to hemodynamic alterations in septic patients. Echocardiography revealed a significant increase in CO of at least 20% in all septic mice 6 hours after CLP. We thus chose to initiate statin therapy 6 hours after sepsis induction by CLP, because at this time point a “clinical” diagnosis based on the changes in hemodynamic status could have been made. Mice were treated with atorvastatin, fluvastatin, pravastatin, simvastatin, or placebo 6 and 18 hours after CLP. No deaths occurred in sham-operated animals. The degree of sepsis induced in CLP operated mice was equal across all groups studied at the time point of treatment (ie, 6 hours after sepsis induction) as assessed by the presence of conjunctivitis, absence of grooming activities with resulting ruffled fur, no oral uptake of food or water, and lethargy. In addition, hyperdynamic cardiovascular states were documented at 6 hours after sepsis induction in subsets of mice from all CLP operated groups. Survival curves and mean survival times for mice in which CLP was performed in Figure 1 clearly delineate the survival benefit sustained from treatment. Compared with 23 ± 1.2 hours of mean survival time in CLP placebo-treated mice, survival was extended by 70% to 39 ± 3.9 hours with atorvastatin, by 74% to 40 ± 4.2 hours with pravastatin, and by 61% to 37 ± 3.6 hours with simvastatin treatment (n=10; P<0.05 versus placebo). Surprisingly, fluvastatin treatment did not result in a significant survival time increase (increase of 17% to 27 ± 2.3 hours; n=10; P=0.759 versus placebo).

Cardiac Function and Hemodynamics Are Preserved by Statin Treatment
To explore changes in CO due to sepsis, we studied conscious mice by echocardiography preoperatively and 20±2 hours after sepsis induction by CLP and evaluated responsiveness to β-adrenoceptor stimulation using dobutamine. Preoperatively, no differences were observed between any of the groups. At 20 hours after CLP (Figure 2A), CO declined from 1.22±0.03 to 0.82±0.03 mL · min⁻¹ · g⁻¹ in CLP placebo-treated mice (P<0.005; n=10) while remaining unaltered in CLP mice treated with atorvastatin (1.26±0.03 mL · min⁻¹ · g⁻¹ preoperatively versus 1.28±0.03 mL · min⁻¹ · g⁻¹ 20 hours after CLP; P=NS; n=10), pravastatin (1.16±0.03 mL · min⁻¹ · g⁻¹ preoperatively versus 1.12±0.02 mL · min⁻¹ · g⁻¹ 20 hours after CLP; P=NS; n=10), or simvastatin (1.20±0.03 mL · min⁻¹ · g⁻¹ preoperatively versus 1.17±0.02 mL · min⁻¹ · g⁻¹ 20 hours after CLP; P=NS; n=10). CLP fluvastatin-treated animals, however, displayed a decline in CO, albeit not to the extent of CLP placebo-treated animals (1.18±0.03 mL · min⁻¹ · g⁻¹ preoperatively versus 0.98±0.03 mL · min⁻¹ · g⁻¹ 20 hours after CLP; P<0.01; n=10). No significant changes between preoperative and postoperative CO were detected in sham-operated animals. After dobutamine stimulation, CO increased in all groups preoperatively. A similar increase was documented after postoperative dobutamine stimulation in sham-operated mice. Placebo-treated CLP mice remained refractory to β-stimulation (Figure 2B). Notably, the responsiveness to dobutamine was restored in CLP mice by treatment with atorvastatin, pravastatin, or simvastatin (Figure 2B), whereas CLP fluvastatin-treated mice showed a dampened response. For all groups in which CO was augmented by dobutamine stimulation, the effect was secondary to a small increase in heart rate and a larger increase in stroke volume (eg, in CLP atorvastatin-treated mice, heart rate increased by 5% and stroke volume by 14% after dobutamine stimulation 20 hours after sepsis induction).

Subsequently, we performed invasive blood pressure measurements in simvastatin- and placebo-treated anesthetized mice (20 hours after CLP) by cannulating the carotid artery. Again, we applied dobutamine as inotropic stimulus and
ETU, an unspecific inhibitor of NOS, to analyze changes in vascular resistance. Mean arterial blood pressure was decreased by 19/110 (P < 0.01; n = 10) in CLP placebo-treated mice compared with sham-operated mice but remained unaltered in CLP simvastatin-treated mice (Figure 3A). Simvastatin treatment had no effect on blood pressure in sham-operated mice (Figure 3A). Dobutamine stimulation led to an increase in arterial blood pressure in sham-operated and in CLP simvastatin-treated mice, whereas CLP placebo-treated mice displayed no significant change in arterial blood pressure (Figure 3A). NOS blockade with ETU led to an 50% increase in mean arterial pressure. However, the absolute value reached by CLP placebo-treated mice remained significantly below the other groups (Figure 3A). This is in concordance with the lower CO in CLP placebo-treated mice within the same time frame but may also correspond to a reduction in peripheral vascular resistance mediated only partly by NO.

**Effects on eNOS Stimulation and Coronary Flow Reserve**

Simvastatin treatment preserved contractility of isolated hearts, which was impaired by 48% in CLP placebo-treated mice compared with sham-operated animals (P < 0.005; n = 10; the Table). Similarly, left ventricular developed pres-


Effects on Leukocyte-Endothelial Adhesion

Absolute leukocyte count was reduced in all mice subjected to CLP compared with sham-operated respective controls (Figure 4A). No differences in leukocyte count were observed between placebo- and statin-treated septic mice with the exception that atorvastatin treatment significantly attenuated the reduction in leukocyte counts compared with placebo in CLP mice (Figure 4A).

To investigate the effects of sepsis and statin treatment on leukocyte-endothelial interaction, mononuclear cells were isolated from septic and sham-operated mice that had received placebo, atorvastatin, fluvastatin, pravastatin, or simvastatin treatment and subjected to adhesion assays on cytokine-stimulated murine endothelial cells under physiological flow conditions. The adhesion of monocytes isolated from placebo-treated CLP mice was significantly increased compared with that of placebo-treated sham operated mice (Figure 4B). Treatment with simvastatin attenuated the enhanced susceptibility to eNOS stimulation in CLP mice. Maximal coronary flow, measured after adenosine application, was similar in all groups. However, coronary flow reserve was severely reduced in CLP placebo-treated mice in which adenosine stimulation resulted in a similar degree of flow increase as observed under bradykinin application.

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In parallel to our previously published in vitro analysis of simvastatin,10 the effect of atorvastatin, fluvastatin, or pravastatin was also evaluated in vitro by treatment of WEHI-274.1 cells and stimulated SV40-immortalized murine endothelial cells with the statins for 30 minutes or 24 hours. After 30 minutes of treatment, the number of adherent cells was reduced when monocytes, endothelial cells, or both were incubated with atorvastatin or fluvastatin (Figure 5A and 5B). 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, suggesting a direct interference with LFA-1 activity in leukocytes or stimulation of eNOS and subsequent antiadhesive effects. This is substantiated by the fact that adhesion of monocytes pretreated with pravastatin (known not to interact with LFA-1) for 30 minutes remained unaltered. Indeed, for

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<th>CLP + Placebo</th>
<th>CLP + Simvastatin</th>
<th>Sham + Simvastatin</th>
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<td>dP/dtmax, mm Hg/s</td>
<td>2654±150*</td>
<td>4562±320</td>
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<tr>
<td>LVDP, mm Hg</td>
<td>73.7±1.9†</td>
<td>97.9±5.5</td>
<td>104±5.4</td>
<td>112.5±4.9</td>
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LVDP indicates left ventricular developed pressure. Values are mean±SEM. CLP placebo-treated mice showed significant contractile impairment (*P<0.005, †P<0.01; sample size, n=10 for all groups), whereas CLP simvastatin-treated mice remained at par with sham-operated mice. Simvastatin treatment alone had no influence on contractile function in sham-operated mice.
pravastatin, treatment of both monocytes and endothelium was necessary to achieve reduced endothelial adhesion (Figure 5C).

After 24 hours, the inhibitory effect of atorvastatin and pravastatin was completely reversible (ie, no longer significantly reduced compared with control) by coincubation with mevalonic acid of monocytes, endothelial cells, or both cell types (Figure 6A and 6B). This indicates that interference with the mevalonic acid–dependent pathway by atorvastatin and pravastatin (eg, inhibition of Rho-GTPase membrane localization and activity) is predominantly responsible for adhesion effects after 24 hours of treatment with the above statins.

Discussion
Following our promising recent study involving pretreatment with simvastatin to improve survival in sepsis,19 the present results give rise to even greater optimism. Significantly improved survival is demonstrated for treatment after the onset of sepsis, a situation more akin to clinical reality, especially because a time point with clinically detectable sepsis effects was chosen for treatment initiation. Furthermore, this benefit is not limited to simvastatin but extends to 2 of 3 additional statins currently evaluated, namely atorvastatin and pravastatin. As with pretreatment, the improvement in survival observed here stems from the complete preservation of cardiac function and hemodynamic stability observed in mice treated 6 hours after sepsis induction with atorvastatin, pravastatin, or simvastatin. Increased mononuclear cell adhesiveness in septic mice, an important contributor to sepsis pathophysiology, is reversed by treatment with all studied statins and represents 1 of the underlying mechanisms.

Because the CLP model of sepsis closely resembles the pathophysiology of human sepsis,26 it provided us with the opportunity to define a time point at which hemodynamic alterations secondary to sepsis induction became apparent. These hemodynamic alterations, namely a hyperdynamic followed by a hypodynamic physiological state, that also typically are found in patients affected by sepsis were readily detectable in our model by serial echocardiographic studies. A hyperdynamic increase in excess of 20% above baseline was observed in all septic mice 6 hours after sepsis induction by CLP; thus, this time point was chosen for the beginning of statin therapy.
In concordance with our previous studies of simvastatin pretreatment in sepsis, impaired CO is secondary to reduced contractility of septic hearts, and this acute septic cardiomyopathy is refractory to catecholamine stimulation, a fact paralleled in human pathophysiology. The reduced arterial blood pressure of septic mice and the limited rise in blood pressure observed after nonselective NOS inhibition suggest that reduced peripheral vascular resistance is mediated by NO and further vasodilatory agents. The pronounced cardiac dysfunction and hypodynamic circulation present severe manifestations of sepsis contributing to the poor outcome and short survival time documented in our mouse model of sepsis. Although not reaching the ∼4-fold increase in median survival time observed in simvastatin-pretreated septic mice, our median survival time was increased by >60% for atorvastatin, pravastatin, and simvastatin treatment. Indeed, therapeutic benefit from these statins was so robust that cardiac function and hemodynamic status remained completely unaffected 20 hours after sepsis induction.

Because TNF-α mimics sepsis by enhancing the endothelial expression of adhesion molecules, chemokines, and cytokines, we isolated peripheral blood mononuclear cells from mouse blood and studied their adhesion to TNF-α-stimulated endothelial cells. As expected, adhesion of monocytes isolated from septic animals was increased. However, monocytes from statin-treated septic animals showed markedly attenuated adhesion to endothelium. 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. Pleiotropic actions of statins include 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. The above-mentioned observations could all be reversed by the addition of mevalonate, indicating that they are causally related to the inhibition of HMG-CoA reductase. However, Weitz-Schmidt et al demonstrated that several statins are capable of blocking the LFA-1–ICAM-1 interaction, providing a mevalonate- and thus HMG-CoA reductase–independent pathway for antiinflammatory and immunomodulatory statin actions. In accordance with these findings, in our in vitro experiments, even a very short incubation of monocytes with atorvastatin and fluvastatin (and simvastatin, as published previously) but not pravastatin (with pravastatin known to differ from atorvastatin and fluvastatin in that it does not interact with LFA-1) resulted in reduced adhesion of monocytes to endothelium not reversible by mevalonic acid. However, other mechanisms such as decreased CD11b expression and reduced CD-11b-dependent adhesion of monocytes on endothelium also demonstrated for simvastatin cannot be excluded. Moreover, effects of lovastatin on β1-integrin–mediated adhesion have been demonstrated. Because both of these effects are reversible with mevalonic acid, an involvement of geranylgeranylated proteins has been postulated. Such mechanisms are indeed more likely because pravastatin was effective in reducing monocyte adhesiveness after 24 hours in a mevalonate-reversible fashion. In addition, the absence of pravastatin effects on monocyte adhesion after 30 minutes of incubation might be interpreted as a pharmacokinetic effect, especially because pravastatin was the only hydrophilic statin studied. Under this hypothesis, we would expect uptake of pravastatin to be delayed compared with more lipophilic statins, so treatment of both monocytes and endothelial cells would be required to achieve the observed significant inhibition by additive effects. This is also supported by the tendency toward reduced adhesion after treatment of endothelial cells alone with pravastatin for 30 minutes. In addition, fluvastatin did not improve survival despite blocking LFA-1–mediated adhesion, so interference with LFA-1 does not seem to be the sole mechanism required to provide effective sepsis therapy with statins.

Incubation of endothelial cells with atorvastatin and fluvastatin (and as previously reported with simvastatin) for 30 minutes also led to a decline in monocyte adhesion. Because this incubation period is too short to modify the cellular sterol pool or the function of Rho, mechanisms operating independently of the cholesterol synthesis pathway must exist. One of these mechanisms could be NO release from endothelium observed as early as 8 minutes after exposure to statins. Indeed, the increase in coronary flow observed after bradykinin stimulation of hearts from sham-operated animals after treatment with simvastatin is likely mediated by increased endothelial NO release. Statins have been described to decrease the phosphorylation of p44 (encoded by ERK1 gene), which is partially enhanced by the addition of mevalonate, while suppressing the phosphorylation p42 (encoded by ERK 2 gene), which is not restored by the addition of mevalonate. Although this mechanism was described in smooth muscle cells, these proteins have also been implicated in endothelial cell responses to shear stress. In light of our experiments, these signal elements might be more prominently involved in endothelial responses to statins than previously appreciated.

In summary, atorvastatin, pravastatin, and simvastatin, well established in the treatment of lipid disorders and coronary artery disease, might have the additional potential of being effective agents in the treatment of sepsis. We believe that the promising results presented here, established in a clinical relevant disease model and applying a clinically feasible therapeutic regimen, in conjunction with abundant safety data resulting from the widespread application of statins and encouraging clinical retrospective and observational data, warrant prospective phase III trials.

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References


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