Hydroxymethyl-Glutaryl Coenzyme A Reductase Inhibition Limits Cytomegalovirus Infection in Human Endothelial Cells

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Background—Statins exert anti-inflammatory effects independently of cholesterol-lowering properties. Cytomegalovirus (CMV) infection appears to be implicated in the pathophysiology of atherosclerosis by inducing inflammatory modifications in endothelial cells, especially in immunosuppressed patients. We investigated whether the activity of statins can inhibit replication of CMV in human endothelial cells.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were infected with CMV and coincubated with fluvastatin at 0.1 and 0.2 μmol/L. Fluvastatin inhibited (P<0.001) CMV antigen expression, and this effect was dose related (P<0.001). Quantitative polymerase chain reaction showed that CMV DNA concentration was consistently lower in supernatants from fluvastatin-treated cells than in infected controls, and viral particle concentration was up to 30 times lower in 0.2 μmol/L fluvastatin-treated cells than in infected controls (10.5±0.9 versus 0.34±0.03 per 10⁷ pfu/mL, P<0.001). Addition of mevalonate to treated cultures almost completely abolished fluvastatin inhibition of viral growth. Electrophoretic mobility shift assay showed that fluvastatin reduced nuclear factor-κB binding activity in CMV-infected cells.

Conclusions—HMG-CoA inhibition by fluvastatin restrains CMV replication in HUVECs by inhibiting viral antigen expression, DNA synthesis, and viral particle production, conceivably by involving a reduction of nuclear factor-κB binding activity. (Circulation. 2004;109:532-536.)

Key Words: statins • atherosclerosis • endothelium • infection • viruses

The inflammatory response of vascular cells to injury is a major pathogenetic mechanism in the development of atherosclerosis.¹ Recent experimental and clinical evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) may act on endothelial inflammatory response, with involvement of cholesterol-independent mechanisms.² These effects appear to be mediated by the capability of statins to reduce production of proinflammatory cytokines and adhesion molecules in activated endothelial cells via interference with signaling pathways that require prenylated proteins.²,³

Intracellular infective agents are among the many possible causes of inflammatory activation of endothelial cells.⁴ Reactivation of cytomegalovirus (CMV), a herpes virus that silently infects as much as 70% of the general population, has been implicated in the initiation of vascular injury,⁵ and relations have been reported between anti-CMV antibody levels and extent of atherosclerosis and rates of coronary events.⁶ In heart transplant recipients in particular, CMV appears to be implicated directly in the pathogenesis of graft atherosclerosis⁷,⁸ and represents an important cause of morbidity and mortality.⁹

The possibility that the activity of statins may interfere with the replication of CMV in human endothelial cells has never been reported. In this study, we sought to elucidate whether HMG-CoA inhibition may impair the kinetics of CMV in endothelial cells by analyzing the yield of filial viral particles, CMV antigen expression, and DNA synthesis during treatment with fluvastatin.

Methods

Cell Cultures and Experimental Conditions
Human umbilical vein endothelial cells (HUVECs) were harvested as described previously.¹⁰ The cells were cultured in collagen-coated flasks (Nunc Brand Products) at 37°C and 5% CO₂, in EBM medium (Clonetics) supplemented with 10% fetal bovine serum, 100 U/mL...
Fluvastatin exerts a biological effect and is tolerated by HUVECs in vitro. However, these experiments were designed to analyze the effect of HMG-CoA inhibition over a 14-day period (to allow analysis of the various steps of CMV kinetics), long-term cell tolerance to different concentrations of fluvastatin was tested previously (Virus yield assay). For this purpose, we continuously incubated HUVECs with fluvastatin 0.1, 0.2, and 0.5 µmol/L for 14 days at 37°C and 5% CO2. Culture medium supplemented with the appropriate concentration of fluvastatin was changed every 72 hours to maintain long-term HUVEC viability, as usually recommended. No cytotoxic effect was detectable on the monolayer of cells treated with fluvastatin 0.1 and 0.2 µmol/L, and as in untreated control cells, ~90% were still viable. On the other hand, fluvastatin 0.5 µmol/L showed a progressive cytotoxic effect, characterized by cell retraction and stretching and trypan blue inclusion. We therefore performed the present experiments using 0.1 or 0.2 µmol/L fluvastatin added to the culture medium. All experiments were repeated with the addition of mevalonate (Sigma-Aldrich) 100 µmol/L, the product of enzymatic conversion of HMG-CoA, to culture medium containing fluvastatin 0.2 µmol/L. All experiments were performed with confluent third- to sixth-passage HUVECs plated in collagen-coated 6-well plates.

**Virus**

The clinically isolated human endotheliotropic CMV strain TB40E was propagated in our laboratory in HUVECs and assayed by viral titer, as described previously. In all experiments, HUVECs were inoculated at a multiplicity of infection of 10.

**Immunofluorescence Staining**

The expression of CMV immediate-early (IE) antigens was determined at 1, 3, 5, 7, and 14 days after inoculation by indirect immunofluorescence staining of the HUVECs. Briefly, at each prespecified time, after gentle trypsinization, cells were counted, and cytocentrifuge slides containing ~104 cells were prepared. Cells were then fixed at −20°C with a 3:1 mixture of methanol/aceton and incubated for 60 minutes at 37°C with either monoclonal mouse reacting with IE1/IE2 gene product, diluted 1:20 (E-13, Argene Biosoft), or monoclonal mouse anti-pp150 (UL32), diluted 1:200 (F3, Abbott Diagnostics Division), as appropriate. Antigens were revealed with rabbit anti-mouse Ig fluorescein conjugate diluted 1:50 (ICN Biomedicals Inc). Finally, cells were stained with Evans blue dye.

**Virus Yield Assay**

Virus yield was determined at day 14 from culture supernatants collected and clarified by centrifugation (1200 rpm for 7 minutes at 4°C). Human embryonic lung fibroblast cultures were then seeded with serial dilutions of these supernatants, and CMV yield was determined as described previously and expressed as plaque-forming units (pfu) per milliliter.

**Quantitative Polymerase Chain Reaction**

Culture supernatants from CMV-infected cells that were either untreated or treated with 0.2 µmol/L fluvastatin with or without mevalonate were collected at days 1, 3, 5, 7, and 14 for quantitative polymerase chain reaction (qPCR). In brief, CMV DNA was extracted from two 100-µL aliquots of each supernatant sample with an Extran nucleic acid extraction kit (Bioline) and then resuspended in 15 µL of RNase-free water. The DNA samples from the 2 aliquots of each supernatant were mixed, and in accordance with the manufacturer’s recommendations, 10 µL of the resulting mixture was used for qPCR (CMV-Iribidoquant, Bioline, Amplimedical s.p.a.). This commercial kit revealed a detection limit of 168 genome equivalents (GE) per milliliter and a 10.2% coefficient of variation.

**Electrophoretic Mobility Shift Assay**

Nuclear factor-κB (NF-κB) activity in CMV-infected HUVECs was compared with CMV-infected cells treated with fluvastatin 0.2 µmol/L and with control uninfected cells by electrophoretic mobility shift assay. Stimulation with tumor necrosis factor-α (TNF-α) for 20 minutes was used as a positive control. Nuclear protein extracts were prepared as described previously. Protein concentrations were determined by the method of Bradford (Bio-Rad Laboratories). Electrophoretic mobility shift assays were performed with the κB motif of the mouse Ig kappa light chain enhancer used as the probe. Equal amounts of nuclear extracts were reacted with 32P-labeled DNA probes encompassing the κB motif of the mouse kappa light chain enhancer. Samples were analyzed on a native 1X Tris-glycine-EDTA polyacrylamide gel. Nuclear extracts were prepared 48 hours after infection and incubated with a 32P-labeled DNA oligonucleotide that contained the recognition site of NF-κB (previous studies indicated the maximum level of NF-κB activation in HUVEC cells to occur between 24 and 72 hours after infection).

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**Table: Effect of Fluvastatin on Antigen Expression and Filial Viral Particle Yield at Different Time Points After Infection**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Untreated HUVECs</th>
<th>MVA</th>
<th>0.1 µmol/L</th>
<th>0.2 µmol/L</th>
<th>ANOVA</th>
<th>0.2 vs Untreated</th>
<th>0.2 vs 0.1 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97±21</td>
<td>95±18</td>
<td>40±12</td>
<td>30±14</td>
<td>0.005</td>
<td>0.029</td>
<td>0.756</td>
</tr>
<tr>
<td>3</td>
<td>84±4</td>
<td>66±17</td>
<td>28±7</td>
<td>32±10</td>
<td>0.033</td>
<td>0.010</td>
<td>0.834</td>
</tr>
<tr>
<td>5</td>
<td>839±35</td>
<td>682±89</td>
<td>464±139</td>
<td>83±25</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>7</td>
<td>1374±454</td>
<td>921±58</td>
<td>577±67</td>
<td>219±84</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.064</td>
</tr>
<tr>
<td>14</td>
<td>9598±241</td>
<td>7750±353</td>
<td>4410±127</td>
<td>1425±117</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Late antigen-positive cells/10⁴

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Untreated HUVECs</th>
<th>MVA</th>
<th>0.1 µmol/L</th>
<th>0.2 µmol/L</th>
<th>ANOVA</th>
<th>0.2 vs Untreated</th>
<th>0.2 vs 0.1 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>105±8</td>
<td>114±8</td>
<td>50±3</td>
<td>38±7</td>
<td>0.033</td>
<td>0.024</td>
<td>0.163</td>
</tr>
<tr>
<td>5</td>
<td>329±21</td>
<td>212±15</td>
<td>168±18</td>
<td>101±9</td>
<td>0.012</td>
<td>0.017</td>
<td>0.071</td>
</tr>
<tr>
<td>CMV yield at 14 days per 10⁶ pfu/mL</td>
<td>10.5±0.9</td>
<td>7.9±1.8</td>
<td>3.8±0.6</td>
<td>0.34±0.03</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

MVA indicates cells treated with fluvastatin 0.2 µmol/L supplemented with mevalonate.
Statistical Analysis
For each separate set of assays regarding the different phases of CMV kinetics (ie, IE and late antigen assay, PCR, virus yield assay, and NF-κB binding activity), at least 3 independent experiments were evaluated. Results are expressed as mean±SD. Differences between groups were assessed by ANOVA with post hoc Bonferroni correction for multiple comparisons. P<0.05 was considered significant.

Results

Antigen Expression
As shown in the Table and Figure 1, untreated HUVECs reached a 100-fold increase in IE antigen expression at day 14 (from 97±21 to 9598±141 positive nuclei/10⁴ cells, respectively; P<0.001). In HUVECs incubated with fluvastatin, IE antigen expression remained significantly lower than in untreated cells throughout the entire experiment, despite a significant time-dependent increase in IE antigen expression among the treated cells. By day 14, IE antigen expression was significantly lower in cells treated with fluvastatin at 0.2 μmol/L than in those treated at 0.1 μmol/L, which suggests a dose-response effect (P<0.001). In all cultures, the late phase of viral kinetics (ie, pp150 [UL32] expression) was observable both at day 3 and day 5. However, late antigen expression was significantly lower in both sets of fluvastatin-treated cells than in untreated cells (Table). The addition of mevalonate almost completely suppressed fluvastatin-related inhibition of IE and late antigen expression.

Viral DNA Production
As depicted in Figure 2, residual viral DNA from CMV inoculation was detectable in culture supernatants at day 1, regardless of treatment (1.6±0.4×10⁶ to 2.5±0.8×10⁶ GE/mL; P=NS). After decreasing at day 3, viral DNA concentration increased much faster in untreated cells than in fluvastatin-treated cells. By day 14, viral DNA concentration was ≈25 times greater in untreated cells (1.5±0.4×10⁸ GE/mL) than in fluvastatin-treated cells (0.6±0.2×10⁷ GE/mL; P<0.01). After supplementation with mevalonate, DNA concentration (1.7±0.4×10⁸ GE/mL) was similar to that detected in untreated cells.

Virus Yield Assay
Presence of infectious CMV particles was sought in supernatants at day 14. As can be seen from the Table, the concentration of CMV particles was approximately 3 to 30 times lower in supernatants from HUVECs incubated with fluvastatin alone (0.1 and 0.2 μmol/L, respectively) than in untreated or mevalonate-supplemented cells.

NF-κB Activity
Figure 3 depicts a representative sample of the results obtained with analysis of NF-κB activity. Although no specific activity was detected in the nonstimulated state (lane 1), both TNF-α and CMV infection induced a strong NF-κB binding activity (lanes 2 and 5). Maximal activation of inducible DNA binding activity was observed in the presence of both CMV and TNF-α stimuli (lane 6). In contrast, TNF-α and CMV-dependent NF-κB activation was inhibited completely when cells were treated with fluvastatin 0.2 μmol/L (lanes 4, 7, and 8). The specificity of the NF-κB binding induced by TNF-α and CMV was confirmed in competition
endothelial cells. In the present study, we therefore investigated the role of fluvastatin in CMV replication and CMV-induced NF-κB response of the host cell (a similar effect has recently been obtained with cerivastatin in cells infected with Chlamydia pneumoniae, although the bacteria vital cycle was not affected by HMG-CoA inhibition24). Despite these considerations, we cannot exclude the possibility that other mechanisms might be involved in the anti-CMV effects of fluvastatin, such as alteration of membrane fluidity or geometry, or the existence of a viral protein that requires prenylation to be activated.25 Although this issue may require further investigation, to the best of our knowledge, such a protein has never been described in CMV. Further studies are required to clarify the role of statins and fluvastatin on CMV replication and the potential therapeutic implications of these findings.

Discussion

This study demonstrates that fluvastatin reduces CMV replication in HUVECs, affecting adult viral particle production, antigen expression, and DNA synthesis. In addition, we found that fluvastatin almost abolished CMV-induced NF-κB activity, which is involved in a pathway that is crucial for CMV replication. To the best of our knowledge, this is the first evidence that statins could inhibit the replication of an infective proatherosclerotic agent in human vascular cells.

After infection, CMV may either complete its replicative cycle and produce adult viral particles or result in a latent/abortive infection, producing only early-phase antigens (a behavior typical of herpes viruses). Although in vitro a complete CMV cycle terminates with cell lysis and dispersion of viral particles in culture supernatants, in vivo studies suggest that production of IE antigens is sufficient to initiate the atherosclerotic process by inducing inflammatory cytokine production, monocyte adhesion, and uptake of LDL in endothelial cells. In the present study, we therefore investigated the possible effects of fluvastatin on each step of the CMV cycle in human endothelial cells, including CMV IE and late antigen expression, viral DNA synthesis, and filial viral particle production during the various steps of productive infection. The concentrations of fluvastatin used in the experiment have been demonstrated not to affect cell viability and were consistent with the in vivo blood concentrations observed during therapy in humans.2

We found that coincubation of HUVECs with fluvastatin strongly inhibited the synthesis of IE and late antigens in CMV-infected cells in a dose-dependent manner (Table; Figure 1). Moreover, qPCR revealed that the concentration of viral filial DNA shed from the cells was up to 25 times lower in fluvastatin-treated HUVECs than in untreated cells (Figure 2). The different time course of fluvastatin on CMV DNA and IE antigen production (Figures 1A and 2) can be ascribed to the different steps of the CMV replicative cycle; whereas IE antigens are nonstructural viral proteins that are produced immediately after CMV enters the host cell, viral DNA is shed from the cell later13 (DNA detected in the early phases of the present experiments is a residual of the virus used for the inoculum). We also detected a dose-dependent reduction of complete filial viral particle yield in the supernatant of fluvastatin-treated HUVECs (Table), which indicates that the generation of completely active CMV particles is reduced in fluvastatin-treated cells. Taken together, these findings indicate that fluvastatin downregulates the entire cycle of CMV replication in endothelial cells. In all 3 sets of experiments, addition of mevalonate to culture medium almost completely abolished the effects of fluvastatin on the various phases of viral replication, which suggests that HMG-CoA activity is crucial to enable CMV replication in endothelial cells.

We also found that fluvastatin treatment curtailed the elevated NF-κB binding activity that is associated with CMV infection (Figure 3). Previous reports2,21,22 showed that HMG-CoA inhibition may limit NF-κB binding activity during proinflammatory stimulation of cultured cells. NF-κB transactivates a large number of proinflammatory cytokines, chemokines, and adhesion molecules, most of which have been implicated in atherosclerosis.2,21,22 Indeed, inhibition of mevalonate synthesis yields a reduced availability of its metabolites, isoprenoids, which are implicated in signaling pathways that require prenylated proteins involved in NF-κB activation.2,21,22 Other studies18,19 showed that CMV requires an increased NF-κB binding activity to initiate and complete its infective cycle, because NF-κB transactivates the CMV IE gene promoter, which is essential for subsequent viral protein synthesis. Moreover, CMV-induced NF-κB activity appears to be the likely trigger of the potentially proatherosclerotic cytokines produced by CMV-infected cells.19 According to this line of evidence, the present data on the fluvastatin-driven limitation of NF-κB binding activity in CMV-infected cells (Figure 3) can provide a plausible explanation for the “antiviral” effect of fluvastatin. Therefore, the present findings further reinforce the concept that NF-κB inhibition might represent a potential therapeutic target to reduce proatherosclerotic stimuli exerted by the inflammatory response of the host cell19,24 (a similar effect has recently been obtained with cerivastatin in cells infected with Chlamydia pneumoniae, although the bacteria vital cycle was not affected by HMG-CoA inhibition24). Despite these considerations, we cannot exclude the possibility that other mechanisms might be involved in the anti-CMV effects of fluvastatin, such as alteration of membrane fluidity or geometry, or the existence of a viral protein that requires prenylation to be activated.25 Although this issue may require further investigation, to the best of our knowledge, such a protein has never been described in CMV.

This study was not designed to explore the role of CMV in atherosclerosis, which has been investigated elsewhere, and our conclusions are limited because they are based only on in
vitro evidence. However, the present data provide a thorough demonstration that HMG-CoA inhibition by fluvastatin may restrain CMV infection in endothelial cells. These results, while confirming the anti-inflammatory properties of statins, identify novel pharmacological targets potentially relevant in improving the therapeutic profile of statins in the treatment of atherosclerosis progression and, possibly, CMV infection development.

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