Hydroxymethylglutaryl Coenzyme A Reductase Inhibitors Modify the Inflammatory Response of Human Macrophages and Endothelial Cells Infected With Chlamydia pneumoniae

H. Kothe, MD; K. Dalhoff, MD; J. Rupp, MD; A. Müller, PhD; J. Kreuzer, MD; M. Maass, MD; H.A. Katus, MD

Background—In patients with atherosclerosis, hepatic hydroxymethylglutaryl coenzyme A reductase (CSE) inhibitors may reduce the activation of inflammation. Because Chlamydia pneumoniae infection has been linked to coronary artery disease through the induction of plaque inflammation, we investigated whether cerivastatin affects the infection rate of human macrophages and endothelial cells (ECs) and their proinflammatory activation after chlamydial infection.

Methods and Results—Macrophages were collected from the alveolar compartment of 6 volunteers and 10 patients with chronic bronchitis. ECs were obtained from 10 umbilical cords. The C. pneumoniae strain CWL was incubated with macrophages or ECs in the presence and absence of the CSE inhibitor cerivastatin. The infection rate was determined by immunofluorescence microscopy. The release of monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), and tumor necrosis factor (TNF)-α was quantified by ELISA. The release of oxygen radicals was determined by ferricytochrome assay. Infection rates were tendentially lower after the preincubation of macrophages with CSE inhibitors (17.2% versus 9.3% and 18.2% versus 10.4%, respectively; P<NS). The secretion of MCP-1, IL-8, and TNF-α by infected macrophages from volunteers increased. Coincubation with cerivastatin resulted in significantly lower MCP-1 and IL-8 production, whereas the release of TNF-α remained unaffected. Similar effects regarding chemokine release were observed in ECs.

Conclusions—CSE inhibitors modify the inflammatory response of human immune cells to C. pneumoniae. This finding could be relevant for the therapeutic potential of CSE statins in patients with atherosclerosis and C. pneumoniae infection. (Circulation. 2000;101:1760-1763.)

Key Words: inflammation ■ endothelium ■ atherosclerosis ■ infection

Chlamydia pneumoniae is an obligate intracellular bacterium that is associated with bronchopulmonary infections and that is capable of multiplying in a wide range of host cells.1 Alveolar macrophages seem to form the major reservoir of pulmonary infection. They respond to challenge with C. pneumoniae by releasing inflammatory mediators,2 and they may serve as a vector for dissemination.3

Inflammation is a major factor in the pathogenesis of atherosclerosis. Atheromatous plaques are characterized by infiltrates of inflammatory cells, which release reactive oxygen species, cytokines, and chemokines. Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) are expressed at increased levels in atheromatous tissue.4 C. pneumoniae is of particular interest as a potential stimulus because viable organisms can be cultured from coronary atheromas.5

Hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors reduce cardiovascular morbidity in patients with atherosclerosis. Growing evidence indicates that statins modulate cellular immune functions, in addition to their cholesterol-lowering effects.6

We investigated the effects of hydroxymethylglutaryl coenzyme A reductase inhibition on infection rate and the production of inflammatory mediators by human macrophages and endothelial cells (ECs) after infection with Chlamydia pneumoniae in vitro.

Culture of C. pneumoniae
Human epithelial cell-2 monolayers containing the C. pneumoniae strain CWL-029 (CWL; ATCC VR-1310) were grown as previously described.2 Chlamydial concentrations of 10 μg/mL and 100 μg/mL were equivalent to 4 or 40 elementary bodies per milliliter, respectively. In preliminary experiments with uninfected human ECs subjected to the same treatment, no mediator release was observed (data not shown).
Infection of Macrophages With CWL
Alveolar macrophages were collected from 6 nonsmoking male volunteers (mean age, 27.2 years) and 10 patients (mean age, 58.4±18 years) with stable, chronic bronchitis. Bronchoalveolar lavage was performed as previously described. The proportion of macrophages was 94.4±2.8% in the volunteers and 93.4±5.1% in the patients. Cells were cultured at a concentration of 10^6/mL in tissue culture plates with medium 199, which contained 5% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin (GIBCO), for 3 hours at 37°C with an air concentration of 5% CO₂. Cells were then washed twice and randomly assigned to coculture with or without CWL (10 to 100 μg/mL) and cerivastatin (0.48 μg/mL).

Cerivastatin was provided by Bayer AG, Leverkusen, FRG. The optimal dosage and timing were determined in other experiments (data not shown). Macrophages from patients with chronic bronchitis were also cocultured with mevalonate (0.13 mg/mL; Sigma).

At 48 and 96 hours, the infected monolayers were methanol-fixed and stained for CWL with a fluorescein isothiocyanate-conjugated antibody (DAKO). Cell viability was assessed before and after culture by trypan blue exclusion.

Infection of Human ECs With CWL
Human ECs were obtained from 10 umbilical cords using the method of Jaffe et al. Cells were suspended in endothelial cell growth medium (Promo Cell), added to culture flasks (Greiner) precoated with fibronectin, and cultured at concentrations of 2×10^5/well at 37°C and 5% CO₂. Purity was assessed by morphology and factor VIII staining. Confluent monolayers from the second passage were cocultured as described above.

Cytokines
Tumor necrosis factor (TNF-α), IL-8, and MCP-1 levels were determined by commercially available ELISAs (Bender and BIOSOURCE) in cell supernatants that were harvested at 48 and 96 hours and stored frozen at −70°C.

Superoxide Anion
Superoxide production was determined by the ferricytochrome C reduction assay (340 ATTC-Photometer, Fa.SLT). This analysis was restricted to supernatants of macrophages from volunteers because of the limited cell numbers obtained with bronchoalveolar lavage.

Nuclear Factor κB Assay
The extraction of nuclear proteins was performed in a subset of 4 patients, with or without CWL (10 μg/mL, 100 μg/mL) coincubation, using the same time points and cell concentrations as for cytokine measurements and according to the "mini extraction method."8

Statistics
Data are expressed as mean±SD and were analyzed with the Wilcoxon test for comparison of nonparametric, paired data and the Mann-Whitney U test for nonparametric, unpaired data. Results were corrected with the Bonferroni adjustment for multiple comparisons. P<0.05 was considered significant.

Ethics
The protocol was approved by the Ethical Committee of the Medical University of Lübeck. All volunteers gave written, informed consent.

Results
Infection Rate of Macrophages and ECs
After the incubation of cells with C. pneumoniae, elementary bodies were readily ingested. Inclusion bodies appeared in macrophages and ECs at 48 hours, indicating productive infection. Macrophages showed a time-dependent mean infection rate between 17.2% and 34% (CWL 10 μg/mL); the infection rate for ECs was between 45% and 60% at 48 and 96 hours, respectively.

Coculture of macrophages with cerivastatin resulted in a nonsignificant reduction of the infection rate (patients without cerivastatin, 18.2±3% versus 10.4±3% with cerivastatin; volunteers: 17.2±5% versus 9.3±4%, respectively); no changes were found after the coculture of ECs with cerivastatin.

Superoxide Anion Production of Macrophages
To evaluate the superoxide anion production, macrophages from volunteers were used. Four hours after infection, superoxide production was significantly increased in infected versus noninfected cells (0.98±0.1 versus 0.63±0.1 nmol/L per 10⁶ cells). Pretreatment with cerivastatin reduced superoxide production by 20.4% in infected cells and by 19% in noninfected cells (P<0.05).

Cytokine Release of Macrophages and ECs
Infection with C. pneumoniae resulted in a significant increase of cytokine release (Table). The relative increase at 96 hours was 7.5-fold for TNF-α, 3-fold for MCP-1, and 2.7-fold for IL-8. Coincubation with cerivastatin reduced the MCP-1 release 3.2-fold and the IL-8 release 1.3-fold (Figure); TNF-α release was not altered.

Macrophages from patients with bronchitis revealed higher spontaneous MCP-1 secretion than macrophages from volunteers, indicating in vivo activation (Table). Levels were not different for IL-8 and TNF-α. Infection of preactivated macrophages with CWL (10 μg/mL) resulted in significant increases of cytokine release. At higher chlamydial concentrations (100 μg/mL), a further stimulation was not detectable. Treatment of activated macrophages with cerivastatin resulted in a reduction of MCP-1 secretion, but the other cytokines remained unaffected. Inhibition of MCP-1 release was completely abolished by mevalonic acid (61 452 pg/mL with mevalonic acid versus 16 438 pg/mL without mevalonic acid, mean of 10 experiments).

Infection of ECs with C. pneumoniae (10 μg/mL) resulted in significant increases of chemokine release (increase at 96 hours was 1.4-fold for MCP-1 and 3.9-fold for IL-8; P<0.05), whereas at higher chlamydial concentrations (100 μg/mL), no further increase was seen. Coincubation with cerivastatin resulted in a 3.2-fold reduction in MCP-1 release (P<0.05) and a 2.8-fold reduction in IL-8 release (P<0.05).

Activation of Nuclear Factor κB
Translocated nuclear factor κB (NFκB) p65 increased significantly in macrophages infected with CWL (10 μg/mL) compared with uninfected cells (1.2±0.3 versus 4.3±0.6 absorbance at 450 nm/nuclear protein; P<0.05), whereas the higher CWL (100 μg/mL) concentration was associated with a reduction of translocated NFκB (3.2±0.6 EU 450 nm/nuclear protein).

Discussion
The major finding of this study was a stimulation of the IL-8 and MCP-1 release of human immune cells by C. pneumoniae.
and an inhibition of the chemokine release after pretreatment with cerivastatin, independent of CWL infection status. These data extend previous findings regarding the release of TNF-α, IL-1, and IL-8 from macrophages and the enhanced MCP-1 production of ECs after chlamydial infection. For these cytokines, a number of proatherogenic effects have been reported. MCP-1 plays an important role in the recruitment of monocytes, and it has been detected in human atheromas. An increased release of MCP-1 by macrophage-derived foam cells and smooth muscle cells was demonstrated. MCP-1 secretion after CWL infection may be due to the activation of NFκB, which increased in parallel with MCP-1 release.

NFκB is a key regulator of a wide range of proinflammatory genes, including TNF-α, IL-8, and MCP-1, and it has been detected in atherosclerotic lesions.

Coculture with cerivastatin reduced the chemokine release from infected and noninfected macrophages and ECs. This effect provides additional evidence for the anti-inflammatory properties of statins, which include an altered regulation of DNA transcription, suppressed natural-killer-cell activity, chemotaxis, enhanced apoptosis, and impaired cytokine production.

In macrophages, the inhibition of MCP-1 release by cerivastatin was not associated with concomitant changes

### Table: Release of MCP-1, TNF-α, and IL-8 in Response to C. pneumoniae Infection and Cerivastatin Pretreatment for 96 Hours

<table>
<thead>
<tr>
<th>Test variable</th>
<th>n</th>
<th>MCP-1, pg/mL</th>
<th>TNF-α, pg/mL</th>
<th>IL-8, ng/mL</th>
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<tbody>
<tr>
<td>Noninfected, nonstimulated macrophages and ECs</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Mac–statin</td>
<td>6</td>
<td>7420±7694</td>
<td>66±80</td>
<td>132±73</td>
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<tr>
<td>Mac + statin</td>
<td>6</td>
<td>1458±2369*</td>
<td>108±149</td>
<td>164±142</td>
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<td>2797±8604</td>
<td>ND</td>
<td>7480±3585</td>
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<tr>
<td>EC + statin</td>
<td>10</td>
<td>3362±2154†</td>
<td>ND</td>
<td>3977±1568</td>
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<tr>
<td>Noninfected, preactivated macrophages and ECs</td>
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<td></td>
</tr>
<tr>
<td>Mac–statin</td>
<td>10</td>
<td>16654±24854</td>
<td>78±60</td>
<td>173±148</td>
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<tr>
<td>Mac + statin</td>
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<td>3461±3783†</td>
<td>67±38</td>
<td>154±186</td>
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<tr>
<td>Infected, nonstimulated macrophages and ECs</td>
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<td></td>
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<tr>
<td>Mac–statin</td>
<td>6</td>
<td>22133±14529‡</td>
<td>477±246</td>
<td>359±179</td>
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<tr>
<td>Mac + statin</td>
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<td>384±211</td>
<td>278±169†</td>
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<tr>
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<td>37547±13451‡</td>
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<td>29432±16129‡</td>
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<tr>
<td>EC + statin</td>
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<td>11929±6834†</td>
<td>ND</td>
<td>10691±6111†</td>
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<tr>
<td>Infected, preactivated macrophages</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Mac–statin</td>
<td>10</td>
<td>42843±71809‡</td>
<td>267±189‡</td>
<td>349±159‡</td>
</tr>
<tr>
<td>Mac + statin</td>
<td>10</td>
<td>16438±29299†</td>
<td>376±217</td>
<td>217±213</td>
</tr>
</tbody>
</table>

Results are mean±SD. Mac indicates macrophage; ND, not done; statin, cerivastatin; –, without; and +, with.

*P<0.01 vs Mac/EC-statin; †P<0.05 vs Mac/EC-statin; ‡P<0.05 vs noninfected Mac/EC.

Mean release of MCP-1 (a) and IL-8 (b) in human alveolar macrophages from 6 volunteers after infection with C. pneumoniae (CWL; 10 μg/mL), with and without coculture with 0.48 μg/mL cerivastatin for 96 hours.
of TNF-α concentrations. These results agree with a previous study, which could not demonstrate an effect of statins on the lipopolysaccharide-induced TNF-α production by monocytes.[10] Alternatively, the inhibition of chemokine release may be induced by a blockade of the mevalonate pathway and a modification of regulatory intracellular proteins.[11] This possibility is supported by the reversal of chemokine inhibition after the addition of mevalonate in our study. The effects of cerivastatin seem to be nonspecific because decreased chemokine release was equally observed in infected and noninfected cells. However, this effect was preserved in situations of cell activation by chronic inflammation (chronic bronchitis) and intracellular infection (C. pneumoniae), which alters multiple cell responses to exogenous stimuli.

In summary, we have demonstrated the stimulation of cytokine release from human immune cells infected with C. pneumoniae and an inhibitory effect of cerivastatin on the chemokine release in vitro. Further studies are needed to evaluate the clinical relevance of these data and the potential therapeutic consequences.

Acknowledgment
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References
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