Hydroxymethylglutaryl Coenzyme A Reductase Inhibition Reduces Chlamydia pneumoniae–Induced Cell Interaction and Activation

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Background—Chlamydia pneumoniae stimulates chronic inflammation in vascular cells. Hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) may have an ameliorating effect. We investigated possible mechanisms.

Methods and Results—We infected human macrophages that in coculture spread infection to vascular smooth muscle cells (VSMCs). Cerivastatin (250 nmol/L) reduced VSMC infection by 33%. Western blotting made it apparent that VSMC infection resulted in increased cell membrane–associated RhoA and Rac1, implying increased prenylation of these proteins. This effect was blocked by statin but circumvented by mevalonate. Cytochrome C assays showed that infected VSMCs produced increased reactive oxygen species that was blocked by statin. Infection increased nuclear transcription factor-κB expression in VSMCs that was dose-dependently suppressed by statin. Infected VSMCs produced and released RANTES and MCP-1. Statin dose-dependently blocked this production both at the mRNA and protein levels. Mevalonate and M geranylgeranylpyrophosphate circumvented these effects.

Conclusions—C pneumoniae can be transmitted from macrophages to VSMCs. VSMCs showed an activation profile typical of atherosclerosis, namely Rac1 and RhoA prenylation, nuclear transcription factor-κB activation, reactive oxygen species production, and chemokine production. Statin reduces macrophage-mediated C pneumoniae–induced signaling and transmission. (Circulation. 2003;108:261-265.)

Key Words: statins ■ Chlamydia pneumoniae ■ atherosclerosis ■ infection ■ immunology

A thomatous plaques harbor inflammatory cells, releasing reactive oxygen species (ROS), cytokines, and chemokines. Monocyte chemoattractant protein (MCP)-1 and “regulated on activation normal T-cell expressed and secreted” (RANTES) are expressed in plaques. Chlamydia pneumoniae may participate in this inflammatory process leading to atherosclerosis, since the organism can be cultured from atheroma.1 We showed earlier that infected vascular smooth muscle cells (VSMCs) show increased nuclear transcription factor-κB expression in VSMCs that was dose-dependently suppressed by statin. Infection increased nuclear transcription factor-κB expression in VSMCs that was dose-dependently suppressed by statin. Infected VSMCs produced and released RANTES and MCP-1. Statin dose-dependently blocked this production both at the mRNA and protein levels. Mevalonate and M geranylgeranylpyrophosphate circumvented these effects.

Methods

C pneumoniae stock suspensions were prepared from the CV-6 strain isolated from an arteriosclerotic coronary artery. Infection was established in primary human VSMC cultures (Clonetics) by centrifuging (2000g, 45 minutes, 35°C) inclusion forming units of the pathogen onto confluent VSMC monolayers in 6-well culture plates.2,3 For infection in the presence of statins, VSMCs were pretreated for 24 hours with cerivastatin (1, 10, 50, and 250 nmol/L) or 10 μmol/L atorvastatin and 100 μmol/L mevalonate, 10 μmol/L geranylgeranyl pyrophosphate, or 10 μmol/L farnesylpyrophosphate 27 hours before infection. The C botulinum C3transferase (10 μg/mL), C difficile toxin B (40 ng/mL), and the specific ROCK-inhibitor Y-27632 (10 μmol/L) were added 3 hours before the statin. Monocytes from a healthy donor were separated with a Ficoll-Histopaque density gradient (Sigma) and subsequent positive selection with anti–CD-14 microbeads (MACS system, Miltenyi Biotec GmbH). CD-14–positive cells were then infected by coincubation with 5 infection-forming units/cell of C pneumoniae strain CV-6 for 72 hours; 10 infected CD-14–positive cells per well were then added onto confluent VSMC monolayers grown in medium with or without 250 nmol/L statin. A centrifugation step was not performed. VSMC infection rates were checked after 96 hours by immunofluorescence microscopy. Experiments were done in triplicate. Homozygous p47phox−/− mice and controls were a gift from Dr Steven M. Holland, Bethesda, Md. VSMCs from mice were isolated by standard procedures. MCP-1, RANTES, IL-6, and IL-8 ELISA were assayed by specific enzyme immunoassays.4 For RT-PCR, RNA was isolated by following the TRIZOL protocol. Quantitative RNA expression was
performed with real-time quantitative RT-PCR, using the TaqMan system. Western blots and electrophoretic mobility shift assay were performed. Band intensities from Western blots and electrophoretic mobility shift assay were analyzed densitometrically by the National Institutes of Health Image Program. RhoA and Rac1 activity are indicated by the amount of membrane-bound normalized to the amount in whole-cell lysates. Statistically significant differences in mean values were tested by 2-way ANOVA for repeated measures and Scheffé’s test. A value of \( P < 0.05 \) was considered statistically significant. The data were analyzed with the use of Statview statistical software.

**Results**

As shown in Figure 1A, macrophages were infected with *C pneumoniae*. VSMCs in monoculture were <1% infected unless a centrifugation-enhancement step was performed, increasing infection to 70%. Statin had no effect on the infection rate. However, the coculture of VSMCs with infected macrophages resulted in a 12% infection rate without the need for additional centrifugation steps. Statin 250 nmol/L reduced the *C pneumoniae* transmission to VSMCs by one third; the experiment was repeated 3 times, and the results were consistent. IL-6 and plasminogen activator inhibitor-1 were detectable at baseline levels (0.6±0.09 and 12.1±1.2 ng/mL, respectively) and increased in coculture to 3.7±0.4 and 35.4±5.4 ng/mL. Preincubation with statin resulted in decreased IL-6 and plasminogen activator inhibitor-1 expression in the supernatant (1.9±0.3 and 18.5±3.7 ng/mL).

We next examined Rac1 and RhoA Figure 1B. Western blotting was performed in infected VSMCs on membrane-bound and cytoplasmic Rac1 or RhoA, with vehicle or statin...
treatment. *C. pneumoniae* increased membrane-bound Rac1 expression that was decreased by statin. The same was observed with RhoA. Also shown are membrane-bound (prenylated) RhoA in noninfected cells (mock), infected cells, statin-treated cells, and statin+mevalonate-treated cells. Infection resulted in a greater expression of membrane-bound RhoA. Statin reduced membrane-bound RhoA. Prenylated RhoA was restored in statin+mevalonate-treated cells. We next examined ROS in infected VSMCs with the cytochrome C assay. Infection sharply increased ROS. Statin decreased this response, which was restored by mevalonate in statin-treated cells.

Figure 2A shows NF-κB activation in vehicle-treated or statin-treated infected VSMCs. NF-κB activation increased by 4 and 24 hours in vehicle-treated cells after infection. Statin treatment reduced NF-κB activation. An IκBα Western blot is shown in the figure. The inhibitory protein was degraded in the vehicle-treated cells but not in the statin-treated infected cells. We next investigated the statin dose response. Statin at low levels decreased the *C. pneumoniae*–induced NF-κB activity. The higher statin doses markedly decreased NF-κB activation. The reduction of NF-κB activity by statins after *C. pneumoniae* infection was counteracted by mevalonate cotreatment. Supershifts for p65 and p50 and competition with unlabeled oligonucleotides corrobated the specificity (data not shown). *C. pneumoniae*–induced NF-κB activity was also reduced in VSMCs from mice lacking the p47 phox protein compared with wild-type mice (Figure 2B). Cotreatment with geranylgeranylpyrophosphate reversed the effect of statins on NF-κB activity, whereas farnesylpyrophosphate had no effect (Figure 2C). AtoRvastatin and Rho GTPase inhibitors reduce *C. pneumoniae*–induced NF-κB activation. *C. difficile* toxin B, which inhibits both Rac1 and RhoA, *C. botulinum* C3 transferase, an exoenzyme, which blocks RhoA, and the specific inhibitor for Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) also ameliorated NF-κB activity (Figure 2C).

We next examined RANTES and MCP-1 expression and release with *C. pneumoniae* infection (Figure 2D). Mock infection had no effect on RANTES or MCP-1. Active infection increased both chemokines in the supernatant. Statin at a high dose abrogated RANTES and MCP-1. We found that 10 and 50 mmol/L statin doses were effective. Finally, we studied the relation between infection, statin treatment, and chemokine expression on the mRNA level. Mock infection had no effect. Active infection increased both RANTES and MCP-1 mRNA, verifying the ELISA (data not shown). This expression was blocked by statin but abrogated by mevalonate. Similar induction was observed for IL-6 and IL-8 (data not shown).

**Discussion**

We found that VSMC infection by cocultivation with infected macrophages was possible, although VSMCs in monoculture were only infected with *C. pneumoniae* by centrifugation pretreatment. Membrane-bound RhoA and Rac1 expression and activity increased in infected VSMCs. Concomitantly, we found that statin diminished the increased VSMC ROS production in infected cells. Rho GTPases and a functional NADPH oxidase were required for *C. pneumoniae*–induced NF-κB activation that was reduced by statins. NF-κB–targeted chemokines reacted accordingly on protein and mRNA levels. These findings support the notion that statins act by inhibiting Rho and Rac prenylation.

Monocyte/macrophages harbor the organisms in a persistent state that is not covered by otherwise effective antibiotic treatment. We observed that the fairly resistant VSMCs could be infected by macrophages in coculture. Spread of infection in this model apparently better reflects physiological processes involved in chlamydial transmission than the artificial centrifugation pretreatment that is required in the absence of monocytes. Therefore, we suggest using the coculture model to analyze immunomodulatory effects on the cross-talk between infected and uninfected cells. Interestingly, statins decreased VSMC infection. Thus, statins may decrease not only the chain of events leading to inflammation and activation in infected VSMCs but also their propensity to become infected.

Laufs et al observed earlier that suppression of endothelial nitric oxide production after withdrawal of statin treatment was mediated by negative feedback regulation of Rho GTPase gene transcription. Blanco-Colio et al found that statins induced VSMC apoptosis by downregulation of Bel-2 expression and Rho A prenylation. Mahony recently showed that Rho family GTPase activity was essential for invasion, since the pan-Rho GTPase inhibitor compactin blocked infection of HEp2 cells.

RANTES activates T cells to proliferate and secrete IL-2, IFN-γ, and MIP1-β in the atherosclerotic plaque. IFN-γ stimulates *C. pneumoniae* to enter the persistent state characteristic of chronic infection. Thus, by stimulating RANTES secretion in VSMCs, *C. pneumoniae* may not only contribute to the trafficking and homing of lymphoid cells but also may induce its own persistence. The RANTES production trigger has not yet been identified. However, our data suggest that *C. pneumoniae* may be involved at an early stage. The infectious hypothesis is further supported by the T-cell profile in plaque lesions. The plaque contains inflammatory infiltrates of CD4+ T cells and macrophages. T cells are activated and secrete Th1 cytokines. These T cells appear polyclonal but are less diverse in the lesions of patients with unstable angina. Furthermore, plaque-derived T cells can be stimulated by *C. pneumoniae*, indicating the presence of pathogen-specific lymphocytes in vivo.

The cerivastatin concentrations we used are attained in vivo when patients receive the higher but clinically relevant 0.4-mg/d dose. We used only human cells in our study, and our *C. pneumoniae* strain was isolated from a human arteriosclerotic plaque. Infected VSMCs produce local factors that can recruit and activate inflammatory cells. Thus, *C. pneumoniae* infection in the vessel wall can induce the cross-talk between cell types by induction of inflammatory cytokines and NF-κB activation. This state of affairs may result in a vicious circle, finally leading to the precipitation of an acute coronary syndrome. Statins interrupt the activated signal transduction cascade of VSMCs after *C. pneumoniae* infection. Our findings support the notion that statins have beneficial effects beyond cholesterol reduction.
Figure 2. A, Influence of cerivastatin on NF-κB activation at 4 and 24 hours after infection in VSMCs. B, VSMCs from p47^−/− mice showed reduced NF-κB activity after infection compared with wild type. C, Cotreatment with geranylgeranyl pyrophosphate abrogated the protective effect on NF-κB activity; farnesylpyrophosphate did not. Influence of various Rho GTPase inhibitors (C botulism 3 transferase exoenzyme, C difficile toxin B, and Y-27632) and atorvastatin on NF-κB after infection is shown. D, Influence of statins on RANTES and MCP-1 protein levels in supernatant of mock and infected VSMCs. The same experiment shows concentration dependence (right).
We are aware that Rho/Rac GTPases not only regulate proinflammatory responses but also cytoskeletal transport mechanisms such as actin filament rearrangement. For example, *Yersinia* and *Brucella* species have active type III secretion systems that inject defined bacterial components into the host cytoplasm, which then regulate phagocytosis and intracellular bacterial transport by modifying Rho activity. Gene deletion and genetic recombination experiments with these facultative intracellular bacteria support this assertion. However, no such experiments have been done with *C. pneumoniae* because of the lack of a system for genetic manipulation of the pathogen.

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**References**

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