Statins Reduce Interleukin-6–Induced C-Reactive Protein in Human Hepatocytes

New Evidence for Direct Antiinflammatory Effects of Statins

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Objectives—Besides its predictive role in determining cardiovascular risk, C-reactive protein (CRP) may exert direct proatherogenic effects through proinflammatory properties. CRP is mainly produced by hepatocytes in response to interleukin-6 (IL-6) and is then released into the systemic circulation. 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors, or statins, significantly reduce cardiovascular events and mortality in patients with or without coronary artery disease and reduce plasma CRP levels in humans. However, the mechanism by which statins reduce plasma CRP levels remains unknown.

Methods and Results—In this study, we report that statins limit both protein and RNA levels of IL-6-induced CRP in human hepatocytes. These effects are reversed by L-mevalonate and mimicked by an inhibitor of the geranylgeranyl-transferase. IL-6–induced CRP production requires the binding of IL-6 to its cognate receptors, which results in activation and phosphorylation of the transcription factor STAT3. We provide evidence that statins reduce this IL-6–induced phosphorylation of STAT3 in hepatocytes.

Conclusion—These results demonstrate that statins reduce IL-6–induced CRP production directly in hepatocytes via inhibition of protein geranylgeranylation. We further show that statins act via inhibition of STAT3 phosphorylation. These findings furnish new evidence for direct antiinflammatory properties of statins and provide new mechanistic insight into their clinical benefits.

Key Words: atherosclerosis • C-reactive protein • inflammation • statins

We currently view atherosclerosis as an inflammatory vascular disease, characterized by accumulation of lipids, fibrous elements, and inflammatory cell infiltrates. Recent clinical trials showed that C-reactive protein (CRP) is a powerful independent predictor of future cardiovascular events. CRP, the major acute-phase reactant in humans, derives mainly from hepatocytes in response to interleukin-6 (IL-6) and is then secreted into the systemic circulation. Recent studies reported that besides its predictive role in determining cardiovascular risk, CRP can exert a direct proatherogenic role. CRP can accelerate the progression of atherosclerosis in apolipoprotein E (apoE)-deficient mice. In vitro studies reporting that CRP modulates the activity and expression of multiple factors implicated in atherogenesis provided further evidence for the proatherogenic role of CRP. CRP downregulates endothelial nitric oxide synthase, resulting in decreased release of NO, and thus facilitates endothelial cell apoptosis and inhibition of angiogenesis. In addition, CRP stimulates the production of the vasconstrictor endothelin-1 and the inflammatory marker IL-6 by endothelial cells. Furthermore, CRP increases the expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1 (MCP-1), resulting in enhanced leukocyte transmigration. Recent data suggest that CRP itself potently chemotacts monocytes and facilitates the uptake of low-density lipoprotein (LDL) by macrophages. In smooth muscle cells, CRP upregulates angiotensin type 1 receptor and stimulates smooth muscle cells migration and proliferation and reactive oxygen species production.

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, effectively lower serum cholesterol levels and significantly reduce cardiovascular events and mortality in patients with or without coronary artery disease. Recently, statins have also been characterized by their immunomodulatory effects, as well as antiinflammatory properties, as demonstrated by their ability to decrease plasma levels of CRP. However, the mechanism by which statins reduce plasma CRP remains unknown.
The signaling pathway that leads to IL-6–induced CRP gene expression in hepatocytes requires the binding of IL-6 to its cognate receptors. Binding of IL-6 to its cell surface receptor, IL-6Rα, induces a formation of complex with the signal-transducing molecule, gp130. The IL-6Rα/gp130 complex further induces the activation and phosphorylation of the JAK kinases, leading to downstream activation of C/EBPβ and STAT3, and thus induction of CRP gene expression.25–28

The aim of this study was to investigate whether statins regulate IL-6–induced CRP production directly at its production site, in hepatocytes, and to highlight the effect of statins on the activation of the transcription factor STAT3 required for IL-6–induced CRP expression.

Methods

Reagents
Human recombinant IL-6 was purchased from R&D Systems (Minneapolis, Minn) and human recombinant CRP was from Calibiochem (San Diego, Calif). Statins were obtained from a commercial source and dissolved in ethanol or H2O (ethanol 100% for atorvastatin, 10% for simvastatin and H2O for pravastatin). L-Mevalonate was from Sigma (St. Louis, Mo). Geranylgeranyltransferase inhibitor (GGTI-298) and farnesyltransferase inhibitor (FTI-277) were obtained from Calbiochem (San Diego, Calif). For CRP enzyme-linked immunosorbent assay (ELISA), capture antibody was purchased from Abcam (Cambridge, UK), and detection antibody was from Dako (Glostrup, Denmark). Anti-IL-6Rα and anti-gp130 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), and anti-STAT3, anti-P-tyr-STAT3, and anti-P-ser-STAT3 were purchased from Cell Signaling Technology (Beverly, Mass).

Cell Culture and Stimulation
Human hepatoma cell line Hep3B (ATCC, Rockville, Md) was maintained in RPMI-1640 medium (Gibco BRL-Life Technologies, Rockville, Md), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO2; primary human hepatocytes were isolated and maintained in culture as previously described.29

Cells were grown to 80% confluence, then washed and incubated in serum-free medium for 24 hours. Cells were then stimulated with 50 ng/mL IL-6 in the presence of different concentrations of statins. Similar concentrations of statin diluents were added to the nonstatin-treated cells for each experiment. To investigate the effect of statins on CRP production by hepatocytes, cells were stimulated with IL-6 for 0 to 48 hours in the presence of different concentrations of statins administered at the same time as IL-6. Then, to study the effects of statins on transcription factor STAT3, cells were first pre-incubated for 4 hours with statins and then stimulated with IL-6 and statins for 15, 30, and 45 minutes.

Analysis of CRP by ELISA
Release of CRP was measured using a sandwich-type ELISA. CRP levels were determined in supernatants of treated cells using optical density measured at 450 nm (Dynaplate reader). The amount of CRP detected was calculated from a standard curve prepared with the recombinant protein. Samples were assayed in duplicate.

RNA Isolation and Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction
Total cellular RNA was extracted with Tri Reagent (MRC, Cincinnati, Ohio) according to the manufacturer’s instructions. To avoid amplification of contaminating genomic DNA, RNA samples were subsequently treated with DNase (Ambion, Huntington, UK). Real-time quantitative reverse-transcription polymerase chain reaction (ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, Calif) was used to determine mRNA levels of CRP. Sequences of the CRP primer/probe combination used: forward primer, 5'-GGG CCC TTC AGT CCT AAT GTC-3; reverse primer, 5'-TTG GCC TGG CAT TTC ATG-3; and probe, FAM-5'-TGTA ACT GCC GGCGAC-3'-TAMRA. Each sample was analyzed in triplicate and normalized in multiplex reaction using TaqMan eukaryotic 18S control (TaqMan Reagent; Applied Biosystems) VIC-labeled.

Western Blot Analysis
Cells were harvested in ice-cold radioimmunoprecipitation (RIPA) lysis buffer. Total protein concentrations were determined using the bicinchoninic acid (BCA) quantification assay (Pierce, Rockford, Ill) and Western blotting was performed as previously described.30 Blots were blocked in 5% dry milk–phosphate-buffered saline (PBS)–0.1% Tween and incubated for 1 hour at room temperature with the following primary antibodies: anti-IL-6Rα, anti-gp130, anti-STAT3, anti-P-tyr-STAT3, anti-P-ser-STAT3, or mouse monoclonal anti-human β-actin as control of loading, followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies. Membranes were developed using an enhanced chemiluminescence system (Pierce) to obtain autoradiograms. After scanning, the blots were analyzed for optical density (National Institutes of Health Image for Windows; http://www.scioncorp.com/pages/scion_image_windows.htm). Values were calculated as percentage of the IL-6–stimulated group, normalized to the β-actin signal.

Statistical Analysis
All results were expressed as mean±SEM. Differences between the groups were considered significant at P<0.05 using the 2-tailed Student t test.

Results
Statins Reduce IL-6–Induced CRP Release in Hepatocytes
We chose the human hepatoma cell line Hep3B as a model to investigate whether statins modulate IL-6–induced CRP production by hepatocytes. In a first set of experiments, cells were stimulated with 50 ng/mL IL-6 in the presence or absence of increasing doses of pravastatin, simvastatin, or atorvastatin. After 24 hours of treatment, CRP levels were determined in the cell supernatants by ELISA. All statins tested significantly reduced IL-6–induced CRP release in a dose-dependent manner (Figure 1A), whereas the strongest inhibition of CRP production was achieved with atorvastatin at a concentration of 100 nM. In addition, 400 μmol/L l-mevalonate reversed the effect of atorvastatin (Figure 1A), which confirms that statins exert their effects by inhibiting the HMG-CoA reductase. L-Mevalonate alone had no effect on the induction of CRP by IL-6 (data not shown). Interestingly, except for pravastatin, the most efficient doses to reduce CRP (1 μmol/L simvastatin and 0.1 μmol/L atorvastatin) correspond to effective serum levels observed with statin treatment in clinical practice.31 To confirm our results on statin-mediated inhibition of CRP production in Hep3B cells, human primary hepatocytes were stimulated for 24 hours with 50 ng/mL IL-6, in the presence or absence of atorvastatin or simvastatin. Human primary hepatic cells treated with 0.1 μmol/L atorvastatin or 1 μmol/L simvastatin also show significant inhibition of IL-6–induced CRP production (Figure 1B). Finally, we measured lactate dehydrogenase (LDH) activity in culture supernatants to evaluate cell necrosis and performed annexin-V staining to evaluate cell
apoptosis. We found no cytotoxic or apoptotic effects either on Hep3B or on primary hepatocytes at any statin concentrations used (data not shown).

Inhibition of Protein Geranylgeranylation, but not Protein Farnesylation, Mimics the Effect of Statins

By inhibiting HMG-CoA reductase, statins reduce the formation of L-mevalonate, as well as further downstream intermediates of the L-mevalonate pathway, such as geranylgeranylpyrophosphate and farnesylpyrophosphate, which represent major intermediates for the post-translational modification of proteins. To study the involvement of protein geranylgeranylation and farnesylation in IL-6–induced CRP production, we investigated whether GGTI-286 and FTI-277, 2 specific inhibitors of the geranylgeranyltransferase or farnesyltransferase, respectively, mimicked the effect of statins. Our experiments revealed that treatment with GGTI-286 significantly reduced IL-6–induced CRP production in hepatocytes, whereas FTI-277 had no effect (Figure 2). These results suggest that the inhibitory effect of statins on IL-6–induced CRP results in part from inhibition of protein geranylgeranylation.

Statins Reduce CRP mRNA Levels in Hepatocytes

To analyze in detail the reduction of IL-6–induced CRP in hepatocytes after statin treatment, we also assessed CRP mRNA expression by real-time quantitative reverse-transcription polymerase chain reaction. First, we performed a kinetic experiment. As shown by Figure 3A, 24-hour stimulation with IL-6 (50 ng/mL) elicited a significant increase in CRP mRNA expression, which was significantly reduced by simvastatin (1 μmol/L), both in Hep3B cell line (Figure 3A) and in human primary hepatocytes (Figure 3B). Similar results were obtained with atorvastatin and pravastatin (data not shown). Furthermore, to avoid an effect of statins on mRNA levels via a modification in degradation/stabilization of CRP mRNA, we performed experiments to determine mRNA stability in the presence or absence of statins. As shown in Figure 3C, we did not observe any modification of CRP mRNA stability in presence of statins, as demonstrated by actinomycin D experiments used to block de novo mRNA synthesis and explore mRNA half-life. These results indicate
expression after statin treatment (Figure 4C).

We further investigated the mechanism underlying the statin-mediated inhibition of CRP. Activation of STAT3, via its phosphorylation, mediates binding to the acute-phase response element to induce CRP gene expression.32 IL-6 phosphorylation, mediates binding to the acute-phase response element to induce CRP gene expression.32 STAT3, and P-tyr-STAT3. Histograms represent the quantification of P-ser-STAT3 and P-tyr-STAT3 after 4-hour simvastatin pretreatment and 15 minutes of IL-6 treatment (Ctrl=control, n=5, *P<0.05 vs IL-6 group). C, Hep3B cells were treated with 1 or 10 μmol/L simvastatin (Smv) for 4, 6, and 8 hours. Expression of gp130 and IL-6Rα was determined by Western blotting. That the reducing effect of statins on CRP mRNA is transcriptional.

**Phosphorylation of STAT3 Mediates IL-6–Induced CRP Expression**

We further investigated the mechanism underlying the statin-mediated inhibition of CRP. Activation of STAT3, via its phosphorylation, mediates binding to the acute-phase response element to induce CRP gene expression.32 IL-6 stimulation of Hep3B cells for different periods revealed that phosphorylation of STAT3 on both tyrosine and serine residues reached maximum levels after 15 minutes (Figure 4A). Pretreatment with 1 and 10 μmol/L simvastatin strongly reduced IL-6–induced phosphorylation of STAT3 on serine residues, whereas we observed no effect on the phosphorylation of tyrosine residues. In addition, statins did not affect the level of STAT3 (Figure 4B). Finally, we demonstrated that the reduction of STAT3 phosphorylation does not result from the reduction of IL-6 receptor expression, because we did not observe any modification of IL-6Rα and gp130 expression after statin treatment (Figure 4C).

**Discussion**

This study demonstrates that statins reduce IL-6–induced CRP expression in human hepatocytes, and that this regulatory effect of statins on CRP expression occurs at the transcriptional level. We further provide evidence that statins act on the geranylgeranyl pathway and reduce CRP gene expression by decreasing the activation of the transcription factor STAT3.

Numerous clinical trials have shown that elevated plasma levels of CRP predicts subsequent acute coronary events among healthy subjects as well as patients with stable or unstable angina.5,33,34 Because CRP, besides its role as an inflammatory marker, is a direct mediator in the development of atherosclerosis,11–13 and because patients who have low CRP levels after statin therapy have better clinical outcomes than those with higher CRP levels, regardless of the resultant level of LDL cholesterol,35,36 therapeutic strategies that reduce CRP levels could be of great interest in the prevention and treatment of cardiovascular diseases. The present study shows, in accordance with previous reports,37 that IL-6 strongly induces the expression of CRP in human hepatocytes, and, for the first time to our knowledge, we demonstrate that statins regulate IL-6–induced CRP expression directly in human hepatocytes (the major site of CRP production). This reduction in CRP expression by statins occurs at the protein and the mRNA levels. We also show that statins have no effect on CRP mRNA stability or degradation, indicating that statins exert their effect at the transcriptional level. In vitro experiments used statins at doses that correspond to those measured in human plasma during therapy, and we observed that according to results in humans, atorvastatin seems to be the most potent inhibitor of CRP production. Many clinical trials have shown that statin treatments reduce plasma levels of CRP. The CARE study has demonstrated that pravastatin reduces CRP levels at 12 and 24 weeks in LDL cholesterol-independent manner.38 Moreover, it seems that atorvastatin exerts a more potent effect on the reduction of CRP than pravastatin, as demonstrated by recent clinical trials.36,39 These results have been confirmed by several studies, with atorvastatin inducing a greater reduction in CRP levels than pravastatin or simvastatin.22 However, although numerous clinical studies have reported that statins lower plasma levels of CRP, our findings demonstrate a direct effect of statins on IL-6–induced CRP expression in human hepatocytes.

Although CRP is mainly produced in response to IL-6, other cytokines, such as IL-1β, can induce this response.41 A very recent study has demonstrated that statins reduce the IL-1β–inducible CRP expression in hepatocytes; however, because IL-1β augments local IL-6 production, this phenomenon may be indirect. In our study, we also show that the statin-mediated reduction of CRP release can be mimicked by GGTI, an inhibitor of protein geranylgeranylation. Thus, the effect of statins on CRP release occurs via the inhibition of protein geranylgeranylation. Induction of hepatic acute phase protein transduction by IL-6 is mainly mediated by the transcription factor STAT3.25,43 The IL-6–mediated activation of STAT3 requires phosphorylation of both tyrosine and serine residues.32,44 The present study shows that statins significantly reduced STAT3 phosphorylation on serine, but not on tyrosine, residues. Two different studies have previously demonstrated that statins reduce IL-6 or angiotensin-
induced phosphorylation of STAT3 in macrophages or in vascular smooth muscle cells, respectively. The signaling pathways leading to STAT3 phosphorylation are well known and differ among tyrosine or serine residues. In the first case, the tyrosine phosphorylation is mediated by the tyrosine kinase activity of stimulated IL-6 receptor complex (IL-6Rα/ gp130/JAK). Our results demonstrate that statin does not influence the IL-6 receptor complex or tyrosine phosphorylation of STAT3. In the other case, recent reports indicate that IL-6–induced serine phosphorylation of STAT3 is mediated by a signal transduction pathway consisting of Vav, Rac-1, MEKK, and SEK-1. The implication of Rac-1 in IL-6–induced STAT3 phosphorylation and transactivation in hepatocytes has been described by several articles. First, it has been shown that IL-6 can activate Rac-1 after 5 minutes of stimulation in hepatocytes. In addition, overexpression of active Rac-1 in hepatocytes strongly enhanced both basal and IL-6–induced STAT3 phosphorylation on serine residue, whereas the dominant-negative Rac-1 had opposite effect.

Thus, from the literature, we can conclude that IL-6 induces CRP release by the following mechanism. Binding of IL-6 to its cognate receptors leads to Rac-1 activation, which in turn induces the phosphorylation of STAT3 on serine residue and then CRP gene expression.

Concerning the effect of statins, we have shown in the present study that statins reduce STAT3 phosphorylation and then CRP gene expression through a decrease of protein geranylgeranylation. Moreover, it has been observed that statins reduce Rac-1 activation in different cell types. Because optimal activation of Rac-1 is dependent on geranylgeranylation, we hypothesized that by suppressing the geranylgeranylation of Rac-1 in hepatocytes, statins reduce IL-6–induced phosphorylation of STAT3, thus resulting in reduced CRP expression (Figure 5).

A growing body of evidence demonstrates that CRP not only is a cardiovascular risk marker but also plays a direct role in the development of atherosclerosis. A recent study using apoE-deficient mice expressing human CRP showed that elevated plasma levels of CRP accelerated the progression of atherosclerosis. Thus, the reduction of CRP expression by statins may contribute to the prevention of cardiovascular diseases. Furthermore, sepsis is often described as a systemic inflammatory response syndrome, and recent studies have shown that statin therapy is associated with a decreased rate of severe sepsis in human and in a murine model of sepsis. Infection elicits acute-phase response, which in turn triggers inflammation and activates the coagulation system. Thus, statins might exert their beneficial effects during sepsis through attenuation of the acute-phase response and its immediate consequences.

In conclusion, our results demonstrate that statins modulate CRP levels by reducing its hepatic gene expression. These findings provide a new molecular explanation for the reduced plasma CRP levels observed in patients treated by statins and thus may help to better understand their great clinical benefits.

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