Statins Decrease Toll-Like Receptor 4 Expression and Downstream Signaling in Human CD14\(^+\) Monocytes

Heiko Methe, Jong-Oh Kim, Sieglinde Kofler, Michael Nabauer, Michael Weis

**Objective**—Anti-inflammatory effects of statins contribute to their clinical benefit. Molecular mechanisms underlying these effects have not been well explored. Because statins attenuate lipopolysaccharide (LPS) responsiveness, we hypothesized that part of the pleiotropic effects are mediated through innate immunity.

**Methods and Results**—Toll-like receptor (TLR) 4 expression and downstream signaling in CD14\(^+\) monocytes after incubation with simvastatin and atorvastatin were quantified via flow-cytometry, quantitative RT-PCR, kinase assay, and enzyme-linked immunosorbent assay. Incubation with intermediates/inhibitors of the mevalonate pathway was used to identify the mode of statin action. Statin incubation resulted in a dose-dependent reduction of TLR4 expression (53±7.6% reduction compared with untreated monocytes; \(P<0.005\)), transcript levels (68±6.3%; \(P<0.002\)), decreased IRAK phosphorylation (37±8.3%; \(P<0.05\)), and LPS-induced IL-6, IL-12, tumor necrosis factor (TNF)-\(\alpha\), and B7-1 expression (\(P<0.05\)). Four weeks of treatment with atorvastatin significantly reduced TLR4 expression on circulating CD14\(^+\) monocytes by 36.2±4.2% (\(P<0.05\)). Effects of statins were reversed by mevalonate (\(P=0.57\)). Incubation with specific inhibitors of geranylgeranyltransferase (54±4.3%), farnesyltransferase (57±5.1%), or with clostridium-difficile toxin B (58±6.1%, \(P<0.01\)) imitated the statin effects. Whereas wortmannin and LY294002 inhibited the statin effect (\(P=0.27\)), incubation with a specific RhoA kinase inhibitor had no effect (\(P=0.57\)).

**Conclusions**—Statins influence TLR4 expression and signaling via inhibition of protein geranylgeranylation and farnesylation. These observations implicate interactions with innate immunity as one pleiotropic mechanism. (*Arterioscler Thromb Vasc Biol. 2005;25:1439-1445*.)

**Key Words:** HMG-CoA reductase inhibitors ■ innate immune system ■ pleiotropic effects ■ Toll-like receptor

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We hypothesized that part of the pleiotropic statin effects are mediated through innate immune mechanisms. The goal of our study was to investigate the impact of statins on expression of TLR4 in CD14\(^+\) monocytes and to explore their effect on TLR4-dependent downstream signaling ex vivo.

**Methods**

**Reagents**

Atorvastatin (ATOR) was a kind gift from Pfizer (Ann Arbor, Mich). Simvastatin (SIM) prodrug (Merck, West Point, Pa) was activated to its active form as described. Mevalonate, geranylgeranylpyrophosphate (GGPP), myelin basic protein, and bacterial LPS (from *Escherichia coli* 0111:B4) were from Sigma-Aldrich (Munich, Germany); \(\text{H}_2\text{O}_2\), geranylgeranyltransferase (GGTase) inhibitor GGTI-298, farnesyltransferase (FTase) inhibitor FTI-277, clostridium difficile toxin B (TcdB), wortmannin, LY294002, RhoA kinase inhibitor HA-1077 were from Sigma-Aldrich (Rockville, Md).

**Monocyte Isolation and Culture**

The study was approved by an institutional review committee and all of the subjects (30 normolipemic healthy volunteers and 10 untreated...
patients with high cholesterol levels) gave informed consent. The clinical and demographic characteristics are shown in the Table.

Blood was drawn from a peripheral vein. Isolation of mononuclear cells was performed by Ficoll density gradient centrifugation. Cells were washed in RPMI1640 medium (Invitrogen GmbH, Karlsruhe, Germany), resuspended in RPMI medium supplemented with 10% calf serum, and plated at a density of 5×10^6 cells per polypropylene tube under rotation to avoid monocyte adhesion and activation. Monocytes were incubated with different concentrations of H2O2. To determine the effect of oxidative stress, monocytes were also incubated with different concentrations of ATOR and SIM for 24 hours in the presence or absence of 1 mmol/L FTI-277, or preincubated with 20 μmol/L GGPP, 200 μmol/L HA-1077, 30 μmol/L FTI-277, or preincubated with 20 μmol/L GGGP, 200 nmol/L wortmannin or 500 nmol/L LY294002 before ATOR treatment. To determine the effect of oxidative stress, monocytes were also incubated with different concentrations of H₂O₂.

In another set of experiments, monocytes were incubated with 30 μmol/L GGTT-298, 400 μmol/L TcdB, 30 μmol/L HA-1077, 30 μmol/L FTI-277, or preincubated with 20 μmol/L GGPP, 200 nmol/L wortmannin or 500 nmol/L LY294002 before ATOR treatment. To determine the effect of oxidative stress, monocytes were also incubated with different concentrations of H₂O₂.

For cytokine analysis and IRAK kinase assay, LPS was added for the time periods indicated. Cell viability was determined by trypan blue exclusion assay. No cytotoxicity could be detected for the applied concentrations.

**In Vivo Effect of Statins**

To assess the in vivo effect of statins on TLR4 expression, 12 normocholesterolemic volunteers were treated with 20 mg ATOR once daily for 4 weeks. Cholesterol levels and surface expression of TLR4 on CD14⁺ monocytes were measured before and after statin treatment by flow cytometry.

mRNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Cultured peripheral blood mononuclear cells were centrifuged and CD14⁺ cells were isolated using MACS CD14⁺ MicroBeads according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). Dynabeads Oligo (dT)25 were used to isolate mRNA according to the manufacturer’s instructions (Dynal, Oslo, Norway). Specific primers were designed using OligoPerfect Designer software (Invitrogen) using sequences accessed through GeneBank and checked for specificity using Blast-search (Table I, available online at http://atvb.ahajournals.org). Real-time polymerase chain reaction (PCR) was performed using the SuperScript III Platinum SYBR-Green One-Step qRT-PCR kit (Invitrogen) following the manufacturer’s instructions. Samples were run in triplicate. Real-time PCR performed on a MX4000-cycler (Stratagene, LaJolla, Calif) was run for 1 cycle (50°C 2 minutes, 95°C 10 minutes), followed immediately by 40 cycles (95°C 15 sec, 60°C 60 sec). Fluorescence was measured after each of the repetitive cycles. For each gene, cycle threshold values were determined from the linear region of the amplification plot. Expression levels of mRNA were normalized to GAPDH mRNA levels. Cytokine and B7-1 mRNA expression levels were presented as the fold increase versus untreated cells. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. To validate the specificity of a primer set, RNA (1 to 3 μg) and a negative control were analyzed in triplicate to confirm that there was no fluorescence resulting from either genomic DNA contamination or from the real-time step. Each PCR performed also

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Values are expressed as mean±SD or N (percentage).

ACE indicates angiotensin-converting enzyme; HDL-chol, high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol; VLDL-chol, very-low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); NS, not significant.
included triplicate wells of no template control in which RNase-free water was added to reaction wells.

Flow Cytometry Analysis
Cells were incubated with TLR4 antibody (mouse anti-human, clone HTA125, IgG3/α; HyCult biotechnology, Uden, the Netherlands) and CD14-PE antibody (mouse anti-human, clone 116, IgM; Beckman-Coulter, Krefeld, Germany) or with mouse IgM IgG2 isotype controls (DakoCytomation, Hamburg, Germany). After washing with staining buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide), polyclonal goat anti-mouse IgG3-fluorescein isothiocyanate (Southern Biotech, Birmingham, Ala) was added. For measurement of B7-1 expression, monocytes were stained with B7-1-fluorescein isothiocyanate antibody (mouse anti-human, clone BB1, IgG3/α; Southern Biotech) or with mouse IgG3/α isotype controls.

Cells were analyzed on a FACScan (Becton Dickinson, San Jose, Calif). Ten thousand CD14+ cells were collected for analysis by Cellquest software (Becton Dickinson). Isotype controls enabled correct compensation and confirmed antibody specificity. TLR4 and B7-1 values were expressed as percentage of CD14+ monocytes.

Immunoprecipitation, Western Blot, and In Vitro Kinase Assay
For immunoprecipitation, Western blot, and IRAK-1 kinase assay, 5x10⁶ cells were collected, pelleted at 1000g for 10 minutes, and lysed on ice for 10 minutes in lysis buffer (50 mmol/L HEPES, pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40, myelin basic protein, 1 g/L). Supernatants for each sample, supplemented with 5 mmol/L ATOR or 10 mmol/L SIM, or RPMI medium (control) for 24 hours. Addition of 1 mmol/L mevalonate completely inhibited the statin effect. Threshold cycle values were normalized for GAPDH expression and presented as mean±SD. *P<0.002 ATOR and SIM vs control and statin plus mevalonate.

Results
In Vitro Effects of Statins on Expression of TLR4 mRNA
First, we investigated the mRNA expression levels of TLR4 in CD14+ monocytes from 30 normolipemic volunteers. Isolated human monocytes were incubated for 24 hours with either 1 mmol/L ATOR or 10 mmol/L SIM. Cells treated with statins had significantly lower levels of TLR4 mRNA (ATOR 0.45±0.29 relative units [RU] versus 1.82±0.58 RU in untreated monocytes; P<0.002, SIM 0.43±0.33; P<0.002). These statin effects were reversed by coincubation with 1 mmol/L mevalonate (ATOR+MEV 1.57±0.53 RU, SIM+MEV 1.52±0.47 RU; Figure 1).

In Vitro Effect of Statins on Protein Expression of TLR4
Protein expression of TLR4 on CD14+ monocytes from 30 normolipemic volunteers after ex vivo coincubation with ATOR or SIM for 24 hours was detected using flow cytometry. Treatment of monocytes with different concentrations of ATOR induced a dose-dependent decrease in TLR4 expression from 35.2±6.6% over 16.8±6.1% (1 mmol/L ATOR; P<0.005) and 8.2±2.7% (10 mmol/L ATOR; P<0.005) to 4.3±4.2% (20 mmol/L ATOR; P<0.001). Similar dose-dependent changes were detected after SIM treatment for 24 hours (5 mmol/L: 14.8±4.2%; P<0.005; 10 mmol/L: 8.1±2.4%; P<0.005; 25 mmol/L: 4.4±2.5%; P<0.001; Figure 2). Addition of 1 mmol/L mevalonate completely inhibited the statin effect on TLR4 surface expression (data not shown). Incubation with statins had no effect on CD14 surface expression.

There were no significant differences of TLR4 expression levels between normolipemic volunteers and untreated patients with high cholesterol levels (35.2±6.6% versus
Inhibition of Protein Geranylgeranylation and Farnesylation Induce Downregulation of TLR4 Expression

Farnesylpyrophosphate and GGPP are important for the post-translational modification of small G proteins of the Ras/Rho family and prenylation is prerequisite for the activation of these proteins. To test whether Rho or Ras proteins play a role in statin-dependent modification of TLR4 expression, monocytes from 30 normocholesterolemic volunteers were incubated with ATOR in the presence of the isoprenoids intermediate GGPP or inhibitors of GGTase and Ftase, respectively. Whereas GGPP alone had no effect on TLR4 expression (37.1 ± 10.5% TLR4+/CD14+ monocytes; P = 0.26 versus native monocytes), coinubation with ATOR blocked the statin effect. In analogy, GGTT-298 mimicked the ATOR effect, and FTI-277 also caused a significant decrease of TLR4 expression (Figure 3).

Clostridium Difficile Toxin B but not HA-1077 Reduce TLR4 Expression in Monocytes

The importance of isoprenylation of Rho proteins for the reduction of TLR4 expression was further substantiated by incubation of monocytes with Clostridium Difficile Toxin B but not HA-1077.

TcdB, a glucosyltransferase that inactivates the Rho subfamily without affecting small G proteins of the Ras family. Treatment of monocytes with 400 pmol TcdB for 24 hours substantially reduced TLR4 expression on monocytes (20.1 ± 3.9% TLR4+/CD14+ monocytes; P < 0.005 versus native monocytes). However, incubation of monocytes with HA-1077, a specific RhoA kinase inhibitor (30 μmol/L for 24 hours), had no effect on TLR4 expression (38.6 ± 8.9; P = 0.24 versus native monocytes; Figure 3).

Treatment with Phosphoinositide 3-Kinase Inhibitors Blocks the Statin Effect on TLR4 Expression

Statins can activate the protein kinase Akt as a downstream effector of the small GTPase Rac. Phosphoinositide 3-kinase signaling is involved in the mechanism of Akt activation by statins. Pretreatment with wortmannin (34.6 ± 6.2% TLR4+/CD14+ monocytes; P = 0.53 versus native monocytes) and LY294002 (32.9 ± 6.2% TLR4+/CD14+ monocytes; P = 0.21 versus native monocytes) significantly blocked the ATOR-induced reduction of TLR4 expression (Figure 3). Incubation of monocytes with phosphoinositide 3-kinase inhibitors alone had no effect on TLR4 surface expression (data not shown).

Atorvastatin Effect on TLR Expression In Vivo

Four weeks of ATOR treatment (20 mg/d) of 12 normocholesterolemic volunteers reduced the frequency of TLR4 expression on CD14+ monocytes by 36.2 ± 4.2% (P < 0.05) as compared with baseline levels and led to a significant reduction of plasma cholesterol, low-density lipoprotein cholesterol levels, and a significant elevation of high-density lipoprotein cholesterol levels (Figure 4).

Downregulation of LPS-Induced IRAK-1 Kinase Activity in Monocytes After Statin Incubation

One early consequence of binding LPS to TLR4 is phosphorylation of IRAK. In 3 independent experiments, we investi-
The effects of statins on cytokine protein expression in CD14⁺ monocytes were analyzed by ELISA (TNF-α, IL-6, and IL-12, and B7-1). Unstimulated monocytes secreted very low baseline levels of these cytokines (Figure 5B). Pre-incubation of monocytes with 1 μmol/L ATOR or 10 μmol/L SIM for 2 hours was followed by a significant decrease in expression and secretion of the cytokines examined.

As assessed by flow cytometry, LPS induced a significant B7-1 expression on CD14⁺ monocytes (21.5±3.9% B7-1⁺/CD14⁺ cells, P<0.005 versus control), whereas pre-exposure to ATOR or SIM significantly decreased B7-1 expression (ATOR+LPS: 6.2±0.3%, P<0.05 versus LPS; SIM 5.8±0.5%, P<0.05, data not shown).

**Discussion**

Multiple experimental and clinical studies support additional activity of statins beyond their serum cholesterol-lowering effects. However, little is known about the mechanisms underlying these anti-inflammatory effects of statins.

TLRs have been shown to be important to the innate immune response, and expression levels of these receptors reflect the sensitivity of immune cells to initiate an immune response. Several reports have indicated regulation of TLR expression by various cytokines and molecules and have linked these observations to a pathogenetic role of TLRs in various diseases.
Here we demonstrate that statins exert direct regulatory effects on TLR4 expression in human monocytes that influences cellular activation. Statins reduce TLR4 surface expression on CD14+ monocytes in vivo and ex vivo in a dose-dependent fashion, causing downregulation of IRAK-1 kinase activity and reduced expression of proinflammatory cytokines and B7-1. Statins have been shown to reduce the level of isoprenoids including GGPP and farnesyl pyrophosphate in various cell types by depleting cellular pools of the precursors, which are substrates for GGTase and FTase, respectively.1,2 In this study, we found that GGPP reversed the effect of ATOR. The importance of isoprenylation of members of the Rho subfamily (Rho, Rac, Cdc42) was further substantiated as a specific GGTase inhibitor and TcdB induced downregulation of TLR4 expression on monocytes. Whereas incubation with a specific RhoA kinase inhibitor had no effect on TLR4 expression levels on monocytes, pre-incubation with inhibitors of the phosphoinositide 3-kinase/protein kinase Akt-pathway (as a downstream effector for proteins of the Ras family and the phosphoinositide 3-kinase/protein kinase Akt pathway) reduced LPS-induced NF-κB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. Atherosclerosis. 1999;147:253–261.


**References**


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