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 geranylgeranylation transferase (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 imply 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

HMG-CoA reductase inhibitors, or statins, inhibit the biosynthesis of cholesterol and associated precursors, which are isoprenoid products of mevalonate.1,2 However, benefits from statin therapy appear to exceed their cholesterol-lowering effect. Statin treatment results in inhibition of NF-κB activity and subsequent reduction of the pro-inflammatory cytokines tumor necrosis factor (TNF)-\( \alpha \) and interleukin (IL)6,8,9 Furthermore, statins inhibit lipopolysaccharide (LPS)-mediated activation of human peripheral mononuclear cells and endothelial cells.6–8 Toll-like receptors (TLRs) were identified as key recognition components of pathogen-associated molecular patterns in mammals. Recently, TLR4 was identified as the signaling receptor for LPS.9 Activation of TLR4 is followed by interaction with MyD88 and Tollip, subsequent autophosphorylation of IRAK, and association with TRAF-6. Finally, activation of NF-κB and members of the extracellular signal-regulated kinase and SAPK/JNK and p38 MAP kinases is followed by upregulation of MHC expression and expression of costimulatory molecules (eg, B7-1 and B7-2) and proinflammatory cytokines (eg, IL-1\( \beta \), IL-6, IL-12, TNF-\( \alpha \)).10–12 Accordingly, several reports suggest an important role of TLR4 in cardiovascular disease.13–15

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.16 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 GGTTI-298, farnesyltransferase (FTase) inhibitor FTI-277, clostridium difficile toxin B (TcdB), wortmannin, LY294002, RhoA kinase inhibitor HA-1077 were from Merck Biosciences (Bad Soden, Germany). All reagents contained <0.125 EU/mL of endotoxin as checked by limulus amebocyte lysate assay (Microbiological Associates, 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 10^6 cells per polypropylene tube under rotation to avoid monocyte adhesion and activation. Mononuclear cells were 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.

**Demographic Characteristics and Biologic Parameters**

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

ACE indicates angiotensin-converting enzyme; HDL-cholesterol, high-density lipoprotein cholesterol; LDL-cholesterol, low-density lipoprotein cholesterol; VLDL-cholesterol, very-low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); NS, not significant.

**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 GenBank 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...
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; HyCult biotechnology, Uden, the Netherlands) and CD14-PE antibody (mouse anti-human, clone 116, IgM; Beckman-Coulter, Krefeld, Germany) or with mouse IgM IgG3 isotype controls (DakoCytometry, 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 isotype antibody (mouse anti-human, clone BB1, IgG3; Southern Biotech) or with mouse IgG3 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, 5×10⁶ 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, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L benzamidine, 5 mmol/L para-nitrophenylphosphate, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL each of aprotinin, leupeptin, and pepstatin). Cell debris was pelleted by centrifugation. Protein concentration in the supernatant was determined using a BioRad protein assay (Bio-Rad Laboratories, Hercules, Calif). Ten thousand CD14+ monocytes were collected, pelleted at 1000g for 10 minutes, and incubated for 2 hours at 4°C. Samples were spun in a microcentrifuge. Equal portions. One portion was solubilized by SDS sample buffer, and pepstatin). Cell debris was pelleted by centrifugation. Protein concentration in the supernatant was determined using a BioRad assay kit. Extracts with equal amount of proteins were used for immunoprecipitation. Five μL of polyclonal anti-IRAK antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) were added to 800 μL isolated cell extracts and incubated at 4°C for 3 hours. Fifty μL of 50% slurry of prewashed protein G–agarose beads (Life Technologies-Invitrogen) were added to each sample, followed by incubation for 2 hours at 4°C. Samples were spun in a microcentrifuge and washed in lysis buffer. Each sample was divided into 2 equal portions. One portion was solubilized by SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membrane (both Life Technologies-Invitrogen), Western blotted with anti-IRAK antibody, and detected with ECL. The other portion was washed with kinase buffer (20 mmol/L HEPES, pH 7.6, 20 mmol/L MgCl₂, 20 mmol/L β-glycerophosphate, 20 mmol/L paranitrophenylphosphate, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L benzamidine). Fifty μL of kinase buffer were added to each sample, supplemented with 5 μmol/L ATP, 1 μg myelin basic protein, 1 μL γ³²P-ATP (Amersham, Buckinghamshire, UK), and incubated at 37°C for 30 minutes. SDS sample buffer was added, and samples were subjected to SDS-PAGE analysis. The gel was dried and exposed to x-ray film. Intensity of the radioactive signal was quantified using a PhosphorImager plate (Molecular Dynamics).

**Measurement of Cytokine Concentration by Enzyme-Linked Immunosorbent Assay**
Supernatants of monocytes were separated by centrifugation and quantified by enzyme-linked immunosorbent assay (ELISA). Commercial ELISA assays detecting IL-6, IL-12, and TNF-α (all R&D Systems, Wiesbaden, Germany) were applied. Supernatants for each individual were stored at −70°C and measured at the same time by the same ELISA to avoid variations of assay conditions.

**Statistical Analysis**
Statistical analyses were performed with JMP software (2002; SAS Institute Inc, Cary, NC). Data were found to be normally distributed and expressed as mean±SD. Comparisons between 2 groups were analyzed by Student t test, and comparisons between >2 groups were analyzed by ANOVA followed by Bonferroni post hoc test. A value of P <0.05 was considered statistically significant.

**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 μmol/L ATOR or 10 μmol/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 coinubation 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 coinubation 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 μmol/L ATOR; P <0.005) and 8.2±2.7% (10 μmol/L ATOR; P <0.005) to 4.3±4.2% (20 μmol/L ATOR; P <0.001). Similar dose-dependent changes were detected after SIM treatment for 24 hours (5 μmol/L: 14.8±4.2%; P <0.005; 10 μmol/L: 8.1±2.4%; P <0.005; 25 μmol/L: 4.4±2.5%; P <0.001; Figure 2). 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.
Figure 2. Dose-dependent effects of atorvastatin and SIM on TLR4 surface expression on CD14⁺ monocytes. After in vitro incubation with RPMI medium (control), ATOR (1, 10, 20 μmol/L), or SIM (5, 10, 25 μmol/L) for 24 hours, monocytes from 30 normocholesterolemic volunteers were stained with anti-TLR4. Ten thousand CD14⁺ monocytes were analyzed and TLR4 surface expression was determined by flow cytometry. Data are expressed as mean percentage±SD of CD14⁺ monocytes. *P<0.005 vs control; †P<0.001 vs control.

37.8±7.8% TLR4⁺/CD14⁺ monocytes; P=0.61). Furthermore, incubation with 1 μmol/L ATOR for 24 hours had the same effect on TLR4 expression levels in both groups (16.8±6.1% versus 16.3±9.3% TLR4⁺/CD14⁺ monocytes; P=0.30; data not shown).

In addition, we analyzed the effect of oxidative stress on TLR4 expression. Incubation of CD14⁺ monocytes with H₂O₂ concentrations ranging from 0.5 to 20 μmol/L had no significant effect on TLR4 expression (20 μmol/L: 32.3±6.8% TLR4⁺/CD14⁺ monocytes; P=0.14 versus native CD14⁺ monocytes). Even more, coincubation of monocytes with ATOR and H₂O₂ did not affect statin-induced downregulation of TLR4 expression (ADOR +20 μmol/L H₂O₂: 18.1±8.2% TLR4⁺/CD14⁺ monocytes; P=0.55 versus ATOR alone; data not shown).

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 isoprenoid intermediate GGPP or inhibitors of GGTase and Ftsase, respectively.

Whereas GGPP alone had no effect on TLR4 expression (37.1±10.5% TLR4⁺/CD14⁺ monocytes; P=0.26 versus native monocytes), coincubation with ATOR blocked the statin effect. In analogy, GGTI-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 TcdB, a glucosyltransferase that inactivates the Rho subfamily without affecting small G proteins of the Ras family. Treatment of monocytes with 400 pmol/L 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±13.1% 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 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-
In Vitro Effects of Statins on Expression of TNF-α, IL-12, and B7-1 mRNA

Quantitative real-time PCR was performed using PBMCs from 30 normocholesterolemic volunteers. Monocytes were exposed to 1 μmol/L ATOR or 10 μmol/L SIM. Unstimulated monocytes contained very low baseline levels of TNF-α, IL-6, IL-12, and B7-1 mRNA. After stimulation with 1 μg/mL LPS for 24 hours, significant amounts of TNF-α, IL-6, IL-12, and B7-1 mRNA were detectable. Addition of ATOR or SIM was found to suppress the LPS-induced transcription of the examined cytokines (P<0.002) and of B7-1 mRNA (P<0.005) in CD14+ monocytes (Figure I, available online at http://atvb.ahajournals.org). Addition of 1 mmol/L mevalonate reversed the inhibitory statin effects.

In Vitro Effect of Statins on Protein Expression of TNF-α, IL-6, IL-12, and B7-1

The effects of statins on cytokine protein expression in CD14+ monocytes were analyzed by ELISA (TNF-α, IL-6, and IL-12 in supernatants) and flow cytometry (B7-1). Unstimulated monocytes secreted low baseline levels of IL-6, whereas TNF-α or IL-12 were not detectable at rest. After stimulation with 1 μg/mL LPS for 24 hours, monocytes were found to express and secrete substantial amounts of TNF-α, IL-6, and IL-12 (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.17,18 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.13,19
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 GGTTase and FTase, respectively. In this study, we found that GGTT reversed the effect of ATOR. The importance of isoprenylation of members of the Rho subfamily (Rho, Rac, Cdc42) was further substantiated as a specific GGTTase 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 pathway (as a downstream effector of the small GTPase Rac) in mediating TLR4 expression. Taken together, our results point toward a pivotal role of the small GTPase Rac in mediating TLR4 expression. Because these molecules play also essential roles in TLR-dependent downstream signaling and serve as targets for physiologic signaling pathways (Rho, Rac, Cdc42), it is speculated that statins interfere with a feedback mechanism already shown for the regulation of TLR expression.

Furthermore, the observed statin effects seem to be independent of cholesterol levels because peripheral circulating monocytes from yet untreated patients with hypercholesterolemia showed comparable TLR4 expression levels and statin-induced downregulation as normolipidemic controls. In addition, TLR4 expression was independent of oxidative stress: incubation of monocytes with H2O2 had no impact on TLR4 expression levels and did not alter the statin effect. Just recently, Asahnoune et al demonstrated involvement of reactive oxygen species in TLR-dependent activation of NF-kB. However, they did not show an effect of oxidative stress on TLR expression itself, thus the exact involvement of oxidative stress in TLR4 signaling is still not characterized.

Previous observations showing that statins are able to suppress oxidized low-density lipoprotein induced NF-κB expression and that oxidized low-density lipoprotein upregulates TLR expression in human macrophages further support our hypothesis of statins as regulators of TLR expression. Our results are also in line with other reports showing reduced LPS-induced NF-κB and cytokine expression during statin treatment in various cell types.

In marked contrast to our results, Boekholt et al described that statins were very effective in carriers of the TLR4 polymorphism Aasp299Gly, shown to be associated with an elevated cardiovascular risk. However, others failed to observe an influence of TLR4 polymorphisms on efficacy of statin treatment, and unaffected LPS signaling in carriers of different mutations in the TLR4 gene has been reported.

There are several potential limitations to the present study. The exact mode of statin action on TLR4 expression remains residual. Further studies are necessary to examine the exact molecular mechanisms underlying statin-dependent TLR4 regulation. It also remains elusive from the current data to what extent inhibitory effects of statins result from TLR4 downregulation.

In conclusion, we could demonstrate that statins influence TLR4 expression and signaling via inhibition of protein prenylation. These observations imply interactions with innate immune mechanisms as a potential mechanism of statins to mediate anti-atherosclerotic effects by reducing proatherosclerotic immune activation.

Acknowledgments
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References


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