Atorvastatin suppresses LPS-induced rapid upregulation of Toll-like receptor 4 and its signaling pathway in endothelial cells

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Wang Y, Zhang MX, Meng X, Liu FQ, Yu GS, Zhang C, Sun T, Wang XP, Li L, Wang YY, Ding SF, Yang JM, Zhang Y. Atorvastatin suppresses LPS-induced rapid upregulation of Toll-like receptor 4 and its signaling pathway in endothelial cells. Am J Physiol Heart Circ Physiol 300: H1743–H1752, 2011. First published February 11, 2011; doi:10.1152/ajpheart.01335.2008.—In the present study, we tested our hypothesis that atorvastatin exerts its anti-inflammation effect via suppressing LPS-induced rapid upregulation of Toll-like receptor 4 (TLR4) mRNA and its downstream p38, ERK, and NF-κB signaling pathways in human umbilical-vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs). TLR4 mRNA expression and its downstream kinase activities induced by LPS alone or atorvastatin + LPS in endothelial cells were quantified using quantitative real-time PCR and enzyme-linked immunosorbent assay. Preincubation of LPS-stimulated endothelial cells with TLR4 siRNA was conducted to identify the target of the anti-inflammatory effects of atorvastatin. Atorvastatin incubation resulted in the reduction of LPS-induced TLR4 mRNA expression, ERK1/2 and P38 MAPK phosphorylation, and NF-κB binding activity. Pretreatment with MEK/ERK1/2 inhibitor PD98059 attenuated atorvastatin + LPS-induced NF-κB activity but had no effect on P38 MAPK phosphorylation. In contrast, pretreatment with P38 MAPK inhibitor SB203580 resulted in upregulation of atorvastatin + LPS-induced ERK1/2 phosphorylation but had no significant effects on NF-κB activity. On the other hand, blocking NF-κB with SN50 produced no effects on atorvastatin + LPS-induced ERK1/2 and P38 MAPK phosphorylation. Moreover, TLR4 gene silencing produced the same effects as the atorvastatin treatment. In conclusion, atorvastatin downregulated TLR4 mRNA expression by two distinct signaling pathways. First, atorvastatin stabilized Ik-Bα, which directly inhibited NF-κB activation. Second, atorvastatin inactivated ERK phosphorylation, which indirectly inhibited NF-κB activation. Suppression of p38 MAPK by atorvastatin upregulates ERK but exerts no effect on NF-κB.

atherosclerosis; inflammation; NF-κB

TREATMENT WITH STATINS, competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase, not only reduces the incidence of cardiovascular events (9) but also improves outcomes in patients with sepsis (1). Besides their cholesterol-lowering effect, statins have anti-inflammatory and immunomodulatory benefits. They can inhibit lipopolysaccharide (LPS)-mediated activation of human peripheral mononuclear cells and endothelial cells (18) and reduce the level of the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin (IL)-6 (23), thereby suppressing vascular inflammation and stabilizing vulnerable plaques (17). However, little is known about the mechanisms responsible for these anti-inflammatory effects. LPS is a unique glycolipid comprising most of the outer leaflet of the outer wall of Gram-negative bacteria, and LPS recognition and signal transmission are among the key events in the host defense reaction against Gram-negative bacteria. LPS may be linked to vascular disease, and low levels of circulating endotoxin in humans and rabbits have been shown to promote the development of atherosclerosis (11).

Toll-like receptors (TLRs) are type-I transmembrane receptors expressed on the cell membrane after LPS stimulation. They are key recognition components of pathogen-associated molecular patterns in mammals. TLR4, the first of the TLRs described, has been the focus of particular interest since its recognition as the receptor for LPS. Activation of TLR4 signal is related to its downstream release of inflammatory cytokines in patients with acute coronary syndrome (12). Deficiency of the LPS receptor TLR4 or MyD88 involved in LPS signaling decreases plaque size and macrophage infiltration (4, 5). It remains unclear, however, whether statins can suppress LPS-induced upregulation of TLR4 and its signaling pathway and what molecular and cellular mechanisms are involved. The present study was undertaken to test the hypothesis that atorvastatin exerts its anti-inflammation effect through suppressing LPS-induced upregulation of TLR4 mRNA and its downstream p38, ERK and NF-κB signaling pathways in human umbilical-vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs).

MATERIALS AND METHODS

Materials. Atorvastatin and ProteoExtract Native Membrane Protein Extraction Kit were supplied by Calbiochem (La Jolla, CA). LPS (Escherichia coli 055: B5) was derived from Sigma (St. Louis, MO). Monoclonal antibodies against P38, phosphor-P38, Ik-Bα and SAPK/JNK were obtained from Cell Signaling (Beverly, MA). Antibodies against phosphor-ERK1/2, ERK, JNK, anti-mouse or rabbit IgG antibody conjugated to horseradish peroxidase (HRP) were derived from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-TLR4 antibody was from R&D Systems (Minneapolis, MN). SB203580 and PD98059 were from BioSource (Camarillo, CA). SN50 was obtained from Alexis Biochemicals (Switzerland) and recombinant human VEGF was from CHEMICON (Temecula, CA).

Cell culture. HUVECs were isolated from fresh umbilical veins by enzymatic disaggregation with collagenase/dispose as described previously (14). HAECs were purchased from Clonetics-Bio Whittaker and ATCC. HUVECs were cultured through three to four passages in

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20% FBS M199 medium at 37°C in a 5% CO2-95% air environment and HAECs (ATCC CA) were cultured in M199 medium containing 5% FBS and endothelial growth supplements, VEGF. HAECs were passaged by using 0.25% trypsin and passages 3–4 were used in all experiments. The medium was changed every other day until the cells became confluent.

In addition, HUVECs and HAECs growing on gelatin-bottomed slide culture to 90% confluency were 1% serum-starved for 24 h. Thereafter, Trypan blue (1 volume per 2.5 volumes of medium) was added to the suspension, and cells were examined for Trypan blue staining. Cell viability was determined by counting an average of 100 cells per field in less than ×400 magnification in four different fields per culture.

**Quantitative real-time PCR.** HUVECs and HAECs were seeded into six-well dishes with a density of 5 × 10^5 cells per well. Cells were exposed to LPS or atorvastatin with different concentrations and stimulated for different periods of time. Total RNA from cells was isolated with use of Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized according to the manufacturer’s instructions for the Reverse Transcription kit (Promega, Madison, WI). Real-time PCR involved use of LightCycler (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instruction. The transcript amount of β-actin was quantified as an internal RNA control. Quantitative values were obtained from the threshold cycle value (Ct), the point when a significant increase of fluorescence was first detected. Experiments were performed in triplicate for each data point. In cell experiments, the primers used for β-actin were 5’-CCTGTACGCCAACA-CAGTGCC-3’ and 5’- ATACTCCTGTTGCTGATCC-3’ (GenBank accession: No. BC004251; annealing temperature, 60°C) and for TLR4, 5’-AGGATGAGGACTGGGTAAGGA-3’ and 5’-CTGGAT-GAAGTGCTGGGACA-3’ (GenBank accession: No. NM-138554; annealing temperature, 58°C).

**Cellular protein extract preparation.** Cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Igepal, and 1× protease inhibitor cocktail (Roche, Mannheim, Germany) for 20 min on ice. Cell lysates were centrifuged at 5,000 revolution/min for 10 min to remove particles. Supernatants containing cellular proteins were collected and stored at −80°C until use.

Cytoplasm and nuclear proteins were extracted from the control and treated cells by Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, the control and treated cells were lysed with the extraction buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% IGEPAL, 40% glycerol, 1× protease inhibitor cocktail (Roche, Mannheim, Germany), and 1× phosphatase inhibitor cocktail (Roche, Mannheim, Germany) for 10 min on ice. Cell lysates were centrifuged at 13,000 g for 10 min at 4°C to remove insoluble material. The supernatants containing cytoplasmic proteins were collected and stored at −80°C until use.

**Cytoplasm and nuclear proteins were extracted from the control and treated cells by Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, the control and treated cells were lysed with the extraction buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% IGEPAL, 40% glycerol, 1× protease inhibitor cocktail (Roche, Mannheim, Germany), and 1× phosphatase inhibitor cocktail (Roche, Mannheim, Germany) for 10 min on ice. Cell lysates were centrifuged at 13,000 g for 10 min at 4°C to remove insoluble material. The supernatants containing cytoplasmic proteins were collected and stored at −80°C until use.

**Fig. 1.** Assessment of cell viability. A: cell survival rate of human umbilical-vein endothelial cells (HUVECs) was measured with Trypan blue exclusion assay. *P < 0.05 vs. the value of control (24 h). †P < 0.05 vs. the value of serum medium (SM) + atorvastatin (Ato). B: cell survival rate of human aortic endothelial cells (HAECs) was measured with Trypan blue exclusion assay. *P < 0.05 vs. the value of control (24 h). †P < 0.05 vs. the value of SM + Ato. Each experiment was repeated for 3 times.

**Fig. 2.** Toll-like receptor 4 (TLR4) mRNA expression in HUVECs and HAECs after LPS stimulation. TLR4 mRNA expression was analyzed by quantitative real-time PCR analysis. A: dose-effect response of LPS stimulation in HUVECs. *P < 0.05 vs. the value of LPS (0 ng/ml). †P < 0.05 vs. the value of LPS (100 ng/ml). B: time-effect response of LPS stimulation in HUVECs. *P < 0.05 vs. the value of LPS (0 min). C: dose-effect response of LPS stimulation in HAECs. *P < 0.05 vs. the value of LPS (0 ng/ml). †P < 0.05 vs. the value of LPS (100 ng/ml). D: time-effect response of LPS stimulation in HAECs. *P < 0.05 vs. the value of LPS (0 min). Each experiment was repeated for 3 times.
treated cells were washed, collected in ice-cold PBS in the presence of phosphate inhibitors, and pelleted at 500 revolution/min, at 4°C for 5 min. Then pellets were suspended in a hypotonic buffer, treated with detergent and centrifuged at 14,000 g for 30 s. The supernatants (cytosolic fraction) were collected, and pellets were resuspended in the lysis buffer. The suspension was centrifuged at 14,000 g for 10 min. Supernatants were saved as the nuclear fractions and frozen at −80°C until use. The protein concentration was determined by MicroBCA (Pierce, Rockford, IL).

Western blot analysis. HUVECs and HAECs were treated with 100 ng/ml LPS for 30, 60, 120, or 240 min, and then the membrane TLR4 protein levels using ProteoExtract were analyzed by Western blotting. HUVECs and HAECs were cultured in six-well plates, and, after cell confluence was reached, media were replaced with fresh 1% serum medium 24 h before the experiment was started. Atorvastatin (1 μmol/l) was added 12 h before cells were stimulated with LPS (100 ng/ml) for 30 min. In another set of experiments, cells were incubated with specific inhibitors for MAPK kinase (MEK)/extracellular regulated kinase 1/2 (ERK1/2) (PD98059, 10 μM), p38 (SB203580, 10 μM), or NF-κB (SN50, 20 μM) for 1 h. PD98059, SB203580, or SN50 were added after cell incubation with atorvastatin for 1 h and 1 h before the addition of LPS. Whole cell lysate (20 μg for p-p38, p-JNK, or p-ERK), 20 μg of membrane-associated proteins extraction (for TLR4), or 20 μg of cytosolic extract (for IκB-α) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen) by electroblotting for 2 h at 60–75 V. The membranes were blocked for 1 h with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) and then incubated with anti-TLR4 (mouse, 1: 300 dilution), anti-IκB-α (rabbit, 1: 1,000 dilution), anti-phospho-JNK (G-7) (mouse, 1: 300 dilution), anti-SAPK/JNK (mouse, 1: 1,000 dilution), anti-phospho-p38 (mouse, 1: 1,500 dilution), anti-p38 (rabbit, 1: 800 dilution), anti-phospho-ERK (mouse, 1: 300 dilution), or anti-ERK (mouse, 1: 500 dilution) antibody overnight at 4°C. HRP-conjugated secondary antibody against the primary antibody was added at room temperature 2 h before washing the blots three times with TBST. Detection involved enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). The protein mass was compared after quantifying the intensity of protein bands by use of Quantity one software (Bio-Rad, Hercules, CA). The experiment was repeated for three times.

NF-κB activity assay. Confluent HUVECs and HAECs were trypsinized and seeded in tissue culture dishes at a density of 2 × 10⁴ cells/cm². After 24 h, the medium was aspirated and replaced with fresh medium alone or containing the indicated concentrations of LPS, atorvastatin, PD98059, SB203580, or SN50. To assess the temporal changes of cell response, cells were pretreated with either LPS alone (100 ng/ml) or with LPS and atorvastatin (1 μmol/l) for different periods of time. The active NF-κB was measured by use of a specific TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instruction. Briefly, the assay is based on an ELISA principle and is more sensitive to detect and quantify NF-κB activation in small amounts of sample than the more traditional electrophoretic mobility shift assay. The activated NF-κB in nuclear extracts was captured by a double-stranded oligonucleotidic probe in a coated microtiter plate and was quantified by spectrophotometric analysis.

Figure 3. TLR4 protein expression in HUVECs and HAECs after LPS stimulation. Membrane TLR4 protein levels were analyzed by Western blotting. A: dose-effect response of LPS stimulation in HUVECs. *P < 0.05 vs. the value of LPS (100 ng/ml). B: time-effect response of LPS stimulation in HUVECs. *P < 0.05 vs. the value of LPS (120 min). C: dose-effect response of LPS stimulation in HAECs. *P < 0.05 vs. the value of LPS (100 ng/ml). D: time-effect response of LPS stimulation in HAECs. *P < 0.05 vs. the value of LPS (120 min). Each experiment was repeated for 3 times.
probe containing the consensus binding sequence for NF-κB. Consequently, NF-κB binding to the target oligonucleotide was detected by incubation with a mutant κB probe provided in the kit was subtracted from the determined values.

**RNA interference.** HUVECs were grown up to 80% confluence. Briefly, cells were transfected with signal silence-negative siCON (Cell Signaling Technology) (ConsiRNA) or signal silence TLR4 siRNA (siTLR4) at 50 nM concentration using Oligofectamine Reagent (Invitrogen) for 24 h, according to the manufacturer’s instructions (22). The sequences for siRNA targeting were as follows: TLR4 sense, 5-CUUUAUCCAACCAGGUGCATT-3 and TLR4 antisense, 5-UGCACCUGGUUGGAUAAGTT-3. The cells were treated with SB-203580 (10 μM), PD98059 (10 μM), or SN50 (20 μM) at the end of 23 h. Then LPS was added at different times. After incubation for 24 h, cells were subjected to analysis.

**Data analysis.** Data were analyzed with SPSS 10.0 for Windows software. All values were expressed as means ± SD. Comparisons between groups involved use of the Student’s t-test (two-tailed) or one-way ANOVA, as appropriate. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**Cell viability assay.** To examine the cell viability under various experimental conditions during this study, Trypan blue staining was performed for HUVECs and HAECS. Both HUVECs and HAECS in control cultures showed a cell survival rate of >95%, which declined to 88% and 89% in HUVECs and HAECS, respectively, after 12 h of exposure to atorvastatin. In the rest of the groups of cells, cell survival rates varied from 77% to 85% after administration of atorvastatin + PD98059, atorvastatin + SB203580, or atorvastatin + SB203580, with or without LPS (Fig. 1, A and B). These results indicated that cell survival rates were high enough after drug administration to allow for quantitative comparisons between different groups.

**LPS-induced TLR4 mRNA and protein expression.** HUVECs and HAECS showed a low level of TLR4 mRNA expression in the absence of LPS stimulation. After stimulation with LPS (100 ng/ml), however, TLR4 mRNA expression showed a rapid increase and reached a maximum at 30 min (Fig. 2, A–D). In contrast, the expression of TLR4 protein showed a slower increase and reached a maximum 2 h after stimulation with LPS (100 ng/ml) in HUVECs and HAECS (Fig. 3, A–D).

**Effect of atorvastatin on LPS-induced expression of TLR4 mRNA.** Pretreatment with atorvastatin (1 μmol/l) before LPS (100 ng/ml) (atorvastatin + LPS) markedly reduced the TLR4 mRNA level induced by LPS. In HUVECs pretreated with atorvastatin (0.01–10.0 μmol/l) for 12 h before the addition of LPS (100 ng/ml), atorvastatin had a dose-dependent attenuating effect on TLR4 mRNA levels, from 0.81 ± 0.12 (0.01 μmol/l atorvastatin; P < 0.05 vs. LPS stimulation), to 0.58 ± 0.16 (0.1 μmol/l atorvastatin; P < 0.05 vs. LPS stimulation), to 0.35 ± 0.13 (1 μmol/l atorvastatin; P < 0.01), and 0.33 ± 0.12 (10 μmol/l atorvastatin; P < 0.01) (Fig. 4A). Similar results were obtained in HAECS (Fig. 4C). The maximal inhibition of TLR4 mRNA expression was obtained after 12 h of exposure to atorvastatin (1 μmol/l) (Fig. 4, B and D).

**Effect of atorvastatin on LPS-induced phosphorylation of MAPKs.** To elucidate the downstream signaling pathways of atorvastatin-induced inhibition of TLR4, the role of MAPK activation was examined by assessing atorvastatin + TLR4 agonist-

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**Fig. 4. TLR4 mRNA expression in HUVECs and HAECS after atorvastatin pretreatment. A: dose-effect response of atorvastatin inhibition in HUVECs. */P < 0.05 vs. the value of Ato (0 μmol/l). †P < 0.05 vs. the value of Ato (1 μmol/l). B: time-effect response of atorvastatin inhibition in HUVECs. */P < 0.05 vs. the value of Ato (0 h). †P < 0.05 vs. the value of Ato (12 h). C: dose-effect response of atorvastatin inhibition in HAECS. */P < 0.05 vs. the value of Ato (0 μmol/l). †P < 0.05 vs. the value of Ato (1 μmol/l). D: time-effect response of atorvastatin inhibition in HAECS. */P < 0.05 vs. the value of Ato (0 h). †P < 0.05 vs. the value of Ato (12 h). Each experiment was repeated for 3 times.**
induced expression of the phosphorylated forms of ERK1/2, p38 MAPK, and JNK. HUVECs and HAECs were cultured with and without atorvastatin (1 μmol/l) for 12 h and then treated with LPS (100 ng/ml) for 5, 15, 30, and 60 min. Atorvastatin pretreatment inhibited the activation of phosphorylated ERK1/2 induced by LPS at 5 min, and the maximal inhibition was achieved at 30 min (Fig. 5, A and D). Similarly, atorvastatin pretreatment attenuated the activation of phosphorylated p38 MAPK induced by LPS at 5 min (Fig. 5, B and E). In contrast, the expression of phosphorylated JNK was increased after LPS stimulation and was not affected by atorvastatin pretreatment (Fig. 5C).

Effect of atorvastatin on NF-κB binding activity in LPS-stimulated endothelial cells. To examine whether atorvastatin inhibited NF-κB activation in LPS-stimulated endothelial cells, HUVECs and HAECs were cultured in medium alone or treated with LPS (100 ng/ml) alone or treated with atorvastatin + LPS. We first examined whether the DNA-binding activity of NF-κB in HUVECs and HAECs was affected by SN50, a cell-permeable peptide derived from the nuclear localization sequence of p50, which inhibits the nuclear translocation of NF-κB. As seen in Fig. 6D, constitutive NF-κB DNA-binding activity in HUVECs and HAECs was significantly inhibited by SN50. Compared with control cells, LPS-treated cells showed enhanced NF-κB binding activity, which reached a maximum 30 min after LPS stimulation but was inhibited in cells pretreated with atorvastatin (P < 0.05) (Fig. 6, A and B).

To correlate NF-κB activation with changes in cytoplasmic protein complex, we performed Western blot to determine the expression level of IκB-α. HUVECs were cultured in medium alone or treated with LPS (100 ng/ml) alone or treated with atorvastatin + LPS. The IκB-α level 15 min after LPS stimulation was similar to that of control cells, but, 30 min after LPS stimulation, rapid disappearance of IκB-α coincided with maximal NF-κB activation. In cells pretreated with atorvastatin, incubation with LPS did not induce any modification in IκB-α levels (Fig. 6C).

Effect of ERK on NF-κB translocation and activation in atorvastatin-inhibited TLR4 signal pathway. Next, we investigated whether there was a causal relationship between ERK1/2 and NF-κB signaling pathways in HUVECs and HAECs. The enhanced NF-κB binding activity induced by LPS was largely ablated in atorvastatin-exposed cells. Blocking ERK activation with PD98059 largely augmented the downregulation effect (P < 0.01) (Fig. 7, A and C). However, the ERK activation was not affected by SN50, an inhibitor of NF-κB translocation to the nucleus (Fig. 8, A and C).

Effect of p38 MAPK on NF-κB translocation and activation in atorvastatin-inhibited TLR4 signal pathway. In contrast to the effect of the MEK/ERK1/2 inhibitor PD98059 on ERK activation with atorvastatin, p38 MAPK inhibitor SB203580 had no inhibitory effect on atorvastatin-exposed cells. Blocking ERK activation induced by LPS (100 ng/ml) at 5, 15, or 30 min and the inhibitory effects of atorvastatin (1 μmol/l) for 12 h in HUVECs. *P < 0.05 vs. the value of LPS (30 min) + Ato. Each experiment was repeated for 3 times.
Fig. 6. LPS-induced effects on NF-κB activity in HUVECs and HAECs after atorvastatin treatment. A: time-effect response of LPS (100 ng/ml) on NF-κB DNA-binding activity in HUVECs and the effect of atorvastatin (1 μmol/l) on LPS-induced activation of NF-κB. OD, optical density. *P < 0.05 vs. the value of LPS (0 min). †P < 0.05 vs. the value of LPS (30 min) + Ato. B: time-effect response of LPS (100 ng/ml) on NF-κB DNA-binding activity in HAECs and the effect of atorvastatin (1 μmol/l) on LPS-induced activation of NF-κB. *P < 0.05 vs. the value of LPS (30 min) + Ato. C: Western blot analysis showing the time course of LPS-induced cytosolic IκB-α loss and its stabilization induced by atorvastatin in HUVECs. *P < 0.05 vs. the value of LPS (0 min). †P < 0.05 vs. the value of LPS (30 min). D: effect of SN50 (20 μM) on NF-κB activity in HUVECs and HAECs. *P < 0.05 vs. the value of control. Each experiment was repeated for 3 times.

Fig. 7. Effects of ERK and P38 on NF-κB activity in HUVECs and HAECs. A: effect of ERK on NF-κB activity induced by PD98059 (PD) (10 μM) added after 11 h of atorvastatin (1 μmol/l) and 1 h before LPS in HUVECs. *P < 0.05 vs. the value of control. †P < 0.05 vs. the value of LPS (30 min). ‡P < 0.05 vs. the value of LPS (3 min) + Ato + PD. B: effect of P38 on NF-κB activity induced by SB-203580 (SB) (10 μM), added after 11 h of atorvastatin (1 μmol/l) and 1 h before LPS in HUVECs. *P < 0.05 vs. the value of control. †P < 0.05 vs. the value of LPS (30 min). ‡P < 0.05 vs. the value of LPS (30 min) + Ato + SB. C: effect of ERK on NF-κB activity induced by PD98059 (10 μM) added after 11 h of atorvastatin (1 μmol/l) and 1 h before LPS in HAECs. *P < 0.05 vs. the value of control. †P < 0.05 vs. the value of LPS (30 min). ‡P < 0.05 vs. the value of LPS (30 min) + Ato + PD. D: effect of P38 on NF-κB activity induced by SB-203580 (10 μM), added after 11 h of atorvastatin (1 μmol/l) and 1 h before LPS in HAECs. *P < 0.05 vs. the value of control. †P < 0.05 vs. the value of LPS (30 min). ‡P < 0.05 vs. the value of LPS (30 min) + Ato + SB. Each experiment was repeated for 3 times.

with the p38 MAPK inhibitor tended to be greater than that obtained in cells exposed to atorvastatin + LPS alone (Fig. 7, B and D). However, the activation of P38 was not affected by SN50 (Fig. 8, B and D).

Effect of p38 MAPK on ERK phosphorylation in atorvastatin-inhibited TLR4. We then determined whether p38 MAPK negatively regulated phosphorylation of ERK1/2 in HUVECs and HAECs. Preincubation with SB-203580 induced marked
increase of phospho-ERK compared with atorvastatin + LPS alone (Fig. 8, A and C), with no change in p38 level in the presence of PD98059 (Fig. 8, B and D).

**Effect of siTLR4 on MAPKs and NF-κB pathway in LPS-stimulated endothelial cells.** We next investigated the effects of TLR4 gene silencing on MAPK signaling, as this pathway played an important role in the regulation of NF-κB activation. As observed with statins, siTLR4 significantly suppressed LPS-enhanced TLR4 expression, ERK and P38 phosphorylation, and NF-κB binding activity in HUVEC (Fig. 9, A and B). TLR4 gene silencing had no suppressive effect on phospho-JNK (data not shown). The addition of PD98059 to LPS siTLR4 further reduced NF-κB binding activity but had no effects on P38 (Figs. 9C and 10A). Similarly, the addition of SB203580 to LPS + siTLR4 markedly enhanced phospho-ERK but had no effects on NF-κB binding activity (Figs. 9C and 10B).

**DISCUSSION**

Results of numerous studies support the additional activity of statins beyond their serum cholesterol-lowering effects. However, to date, the anti-inflammatory mechanism by which atorvastatin acts on endothelial cells has not been fully explored with regard to the intracellular signaling pathway. The present study showed for the first time that atorvastatin exerted its anti-inflammatory effects by attenuating LPS-induced rapid TLR4 mRNA expression, which led to inhibition of two distinct downstream signaling pathways: inhibition of NF-κB translocation through IkBα stabilization and inactivation of phosphorylation of ERK. Moreover, this study demonstrated a marked inhibition of P38 phosphorylation by atorvastatin although such an inhibition had no significant effects on NF-κB.

TLRs, a family of phylogenetically conserved receptors, have attracted particular interest because of their important functions in regulating and linking immune and inflammatory processes. TLRs are expressed in various cells, including HUVECs and HAECs. Upon LPS stimulation, TLR4 activates NF-κB and MAPK via a signal transduction process involving MyD88 and IL-1 receptor-associated kinases essential for pro-inflammatory proteins (IL-6 and IL-8) (3). Thus upregulation of TLR4 expression may play an active role in inflammatory disease.

Statins have important anti-inflammatory effects in addition to their lipid-lowering effects. Previous studies showed that statins attenuated endotoxin-induced inflammation by inhibiting NF-κB and cytokine expression (7) but did not reveal the molecular mechanisms. Recent studies have demonstrated that
NF-κB plays a critical role in modulating the expression of genes involved in inflammatory responses, cell adhesion, cell cycle and cell survival, and apoptosis in multiple tissues. Because stimulation of TLRs in many cell types has been associated with activation of NF-κB, we investigated whether atorvastatin inhibited NF-κB activation in LPS-stimulated endothelial cells. LPS, a well-known NF-κB-activating inflammatory factor, induced NF-κB DNA-binding activity in endothelial cells, which was inhibited by pretreatment with atorvastatin. Although previous studies showed that statins suppressed NF-κB expression induced by oxidized low-density lipoprotein, which was found to upregulate TLR expression in human macrophages (13), our studies provided direct evidence that atorvastatin acts as a regulator of NF-κB expression via TLR4.

The key step in NF-κB regulation is the translocation of activated NF-κB from the cytoplasm to the nucleus. In most cell types, NF-κB is present constitutively in the cytosol in a latent, inactive form where it is retained through its interaction with inhibitory IκB (inhibitor of NF-κB) proteins, masking its nuclear localization sequence. A variety of stimuli induce phosphorylation of IκB at 2 N-terminal serine residues, followed by ubiquitination and degradation of IκB by the proteasome Rel/NF-κB complex. This complex enters the nucleus, binds to DNA, and activates transcription of target genes whose products include cytokines, chemokines, major histo compatibility complex molecules, proteins involved in antigen presentation, and receptors required for neutrophil adhesion and transmigration across blood vessel walls (21). In the present study, we found that the IκBα level was the lowest 30 min after LPS stimulation which coincided with the maximal NF-κB activation. In cells pretreated with atorvastatin, however, the IκBα level remained normal 30 min after LPS stimulation. These results suggest that atorvastatin may block NF-κB activation by stabilizing IκBα in the cytoplasm.

Previous studies have implicated activation of MAPKs and NF-κB in mediating the effects of specific TLRs in various cell types. To elucidate the signaling pathways in the downstream atorvastatin-induced inhibition of TLR4, we studied the role of MAPK activation by assessing atorvastatin LPS-induced expression of the phosphorylated forms of ERK1/2, p38 MAPK, and JNK. Recent studies reported that MAPK signaling pathways were associated with the vascular inflammation modulated by reactive oxygen species (8). Activation of MAPK proteins is critical in the cellular responses associated with inflammatory stimuli such as LPS (10). Our results showed that LPS upregulated TLR4 and elicited activation of the MAPK and NF-κB signaling cascade in HUVECs and HAECs, which was consistent with previous studies (2). It should be noted that, in the present study, the maximal level of TLR4 mRNA expression was at 30 min, whereas that of TLR4 protein expression was at 120 min in endothelial cells after LPS.
B binding activity by atorvastatin decreased NF-
B translocation to the nucleus. Thus the
activation of ERK was not affected by SN50, an
activation with PD98059 largely augmented the effect. How-
largely ablated in atorvastatin-exposed cells. Blocking ERK
The enhanced NF-
B binding activity induced by LPS was
demonstrated that atorvastatin exerted an inhibitory effect on
inhibitor of NF-
B translocation and MAPK activa-
tion were produced by attenuating TLR4 mRNA expression.
Because both ERK1/2 and NF-κB play important roles in
atorvastatin-induced signaling pathways, we next investigated
the potential causal relationship between these two pathways. The enhanced NF-κB binding activity induced by LPS was
largely ablated in atorvastatin-exposed cells. Blocking ERK
activation with PD98059 largely augmented the effect. How-
ever, the activation of ERK was not affected by SN50, an
inhibitor of NF-κB translocation to the nucleus. Thus the
decreased NF-κB binding activity by atorvastatin + PD98059 is due at least in part to an effect on ERK.
In the present study, we found that suppression of p38 MAPK
by atorvastatin upregulates phosphorylation of ERK1/2. Preincu-
bation with SB-203580 induced a marked increase of phospho-
ERK compared with atorvastatin + LPS treatment alone, with
no change in level of p38 in the presence of PD98059.
Although the mechanisms underlying this inhibitory action of
statins on p38 MAPK remains to be elucidated, one-way cross
stalk between p38 MAPK and ERK1/2 was recently
inhibited, whereby phosphorylated p38α was found to couple
with ERK1/2 and thereby sterically block ERK1/2 phosphory-
lation by MEK1/2 (25) and possibly also act via a protein
kinase that lies upstream of MEK1/2 (19).
The effect of statins on the activity of P38 has been in
spite. Several reports showed that statins downregulated the
activity of P38 (16, 24), whereas other studies reported sus-
tained P38 activation even with statin treatment (20). Our data
demonstrated that atorvastatin exerted an inhibitory effect on
LPS-induced phosphorylation of P38. However, such an inhi-
bition had no effect on NF-κB binding activity, and pretreat-
ment of cells with SN50 had no effect on phosphorylation of
p38 MAPK. These results suggest that there is no cross talk
between NF-κB and P38 signaling pathways and that the role
of P38 signaling pathway in the anti-inflammatory mechanisms
of atorvastatin is probably minimal.
A notable finding of our study was that atorvastatin down-
regulated TLR4 mRNA expression by two distinct signaling
pathways. First, atorvastatin stabilized Iκ-Bα, which directly inhibited NF-κB activation. Second, atorvastatin inactivated
ERK phosphorylation, which indirectly inhibited NF-κB acti-
vation. To further validate our findings, TLR4 gene silence was
conducted, and the results were similar to those of atorvastatin
prior to exposure to LPS for 30 min. The effect of TLR4 gene silence on NF-κB DNA-binding activity was mediated by MAP kinase
in the same way as atorvastatin treatment. These results con-
firm that both atorvastatin and TLR4 gene silence act on the
same target of TLR4 mRNA, which leads to similar changes of
intracellular early signaling pathways.
Our study contained several limitations. First, the chronic
effects of atorvastatin on TLR4-mediated signaling pathways
were not investigated. Recent studies have demonstrated that
statins are able to suppress expression of TLR4 protein in
human macrophages (15) and that LPS-induced NF-κB activity
may have a positive feedback on TLR4 protein expression via
posttranscriptional mechanisms (6). These results support the
notion that inhibition of LPS-induced rapid events by atorva-
statin may ultimately control expression of TLR4 protein itself.
Second, pharmacological inhibitors were used to block signal-
pathways in this study, but these drugs lack sufficient
specificity for given molecules. Combined gene interference is
more preferred approach to specifically block signaling path-
ways, but, unfortunately, combined gene interference of TLR4
and p38 MAPK or that of TLR4 and ERK1/2 resulted in a very

Fig. 10. Effects of siTLR4 on the relation among NF-
κB, ERK, and p38 pathways in HUVECs after atorva-
statin treatment. A: effect of blocked NF-κB and ERK
on the activity of p38 induced by siTLR4 in HUVECs.
*P < 0.05 vs. the value of consiRNA. †P < 0.05 vs. the
value of LPS (30 min) + ConsiRNA. ‡P < 0.05 vs. the
value of LPS (30 min) + siTLR4. B: effect of blocked
NF-κB and P38 MAPK on the activity of ERK induced
by siTLR4 in HUVECs. *P < 0.05 vs. the value of consiRNA. †P < 0.05 vs. the value of LPS (30 min) +
ConsiRNA. ‡P < 0.05 vs. the value of LPS (30 min) +
siTLR4. Each experiment was repeated for 3 times.
low cell survival rate and thus was not adopted in the present study.

In conclusion, the present data demonstrate for the first time that atorvastatin downregulated TLR4 mRNA expression by two distinct signaling pathways. First, atorvastatin stabilized Iκ-Bα which directly inhibited NF-κB activation. Second, atorvastatin inactivated ERK phosphorylation, which indirectly inhibited NF-κB activation. Suppression of p38 MAPK by atorvastatin upregulates ERK but exerts no effect on NF-κB. These findings may contribute to understanding the molecular mechanisms by which statins induce anti-inflammation in HUVECs and HAECS. However, future studies in vivo are required to confirm the molecular mechanisms proposed.

REFERENCES


