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Article

Atorvastatin suppresses lipopolysaccharide-induced inflammation in human coronary artery endothelial cells

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The present study was designed to examine the effects of atorvastatin on vascular inflammatory responses in human coronary artery endothelial cells (HCAECs), when challenged by lipopolysaccharide (LPS), a Toll-like receptor-4 (TLR4) ligand. HCAECs were pretreated with atorvastatin and induced by LPS. The expression of TLR4, interleukin -6(IL-6), monocyte chemoattractant protein 1(MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecular-1(ICAM-1), nuclear factor-κB (NF-κB) and p38 mitogen activated protein kinase(p38 MAPK) were evaluated using Real-time polymerase chain reaction, cytokine ELISA assay and Western blotting. The results showed that pretreatment with atorvastatin down-regulated the expression of TLR4 in LPS-activated HCAECs. Atorvastatin also attenuated the LPS-induced expression of interleukin IL-6 and MCP-1, at both the transcription and translation level in HCAECs. LPS-induced endothelial cell adhesion molecules, ICAM-1 and VCAM-1 expression were also reduced by pretreatment with atorvastatin. Furthermore, atorvastatin efficiently suppressed LPS-induced phosphorylation of NF-κB and p38 MAPK in HCAECs. These findings show that atorvastatin suppresses endothelial cell inflammation, suggesting that atorvastatin may be suitable for development as a therapeutic agent for inflammatory cardiovascular disease.

Keywords: Atorvastatin. TLR4. Lipopolysaccharide. Endothelial cells. Inflammation.

INTRODUCTION

The inflammatory response of vascular endothelial cells plays important roles in the development and progression of atherosclerosis (Kinlay, Ganz, 1997; Simon *et al.*, 1999; Akira *et al.*, 2006). When stimulated by pathogenic mediators, endothelial cells trigger critical inflammatory responses, including enhancement of endothelial permeability, secretion of cytokines/chemokines and recruitment of circulating leukocytes (Raetz, Whitfield, 2002).

Toll-like receptors (TLRs) are type-I transmembrane receptors expressed on the cell membrane. TLR4, the first of the TLRs described, has been the focus of particular interest since its recognition as the receptor for lipopolysaccharide (LPS) (Akira *et al.*, 2001). LPS, a major structural portion of the outer membrane of Gram-negative bacteria, is a ligand and potent agonist of TLR4 (Wiedermann *et al.*, 1999). Activation of the TLR4 signal is related to its downstream release of inflammatory cytokines in patients with acute coronary syndrome (Methe *et al.*, 2005). Endothelial cells, upon LPS stimulation, initiate the over-production of inflammatory cytokines including IL-6, chemokines such as MCP-1, ICAM-1 and VCAM-1 (Yamagami *et al.*, 2003; Dauphinee and Karsan, 2006). Accordingly, several reports have suggested the activation of

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TLR4 plays an important role in the development and progression of some inflammatory diseases (Schroder, Schumann, 2005; Gribar *et al*., 2008). Thus, TLR4 is an excellent therapeutic target for the treatment of inflammatory disease.

Atorvastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA coenzyme A reductase, inhibits the biosynthesis of cholesterol and associated precursors, which are the isoprenoid products of mevalonate (Sinensky *et al*., 1990; Takemoto, Liao, 2001). Besides its cholesterol lowering effects, atorvastatin has anti-inflammatory and immunomodulatory benefits. Atorvastatin inhibits LPS-mediated activation of human peripheral mononuclear cells and endothelial cells (Rice *et al*., 2003) and it suppresses vascular inflammation and stabilizes vulnerable plaques (Pleiner *et al*., 2004). Atorvastatin down-regulates the expression of TLR4 and reduces levels of the pro-inflammatory cytokine, the tumor necrosis factor β (TNF-β) and IL-6 (Weis *et al*., 2001). HCAECs, an endothelial cell line derived from human coronary artery endothelial cells, are commonly used for *in vitro* experimental models of vascular endothelial cells (Liu *et al*., 2003; Dela Paz *et al*., 2017). However, little is known about mechanisms of atorvastatin response for these anti-inflammatory effects in HCAECs.

In this study, we investigated the effects of atorvastatin on the expression of LPS-induced inflammatory genes (IL-6 and MCP-1) and adhesion molecules such as ICAM-1 and VCAM-1 in HCAECs. We also analyzed the LPS-induced phosphorylation of the signaling molecules, NF-κB and p38 MAPK.

MATERIAL AND METHODS

Material

Atorvastatin was supplied by Calbiochem, China. Human Coronary Artery Endothelial Cells was supplied by Cell Applications, China. LPS (Escherichia coli 055: B5), protease inhibitor cocktail, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, insulin-like growth-factor, ascorbic acid, epidermal growth factor, heparin and other chemicals, unless otherwise stated, were purchased from Sigma, China. Monoclonal antibodies against p38, phosphor-p38, ICAM-1, VCAM-1 and β-actin were purchased from Cell Signaling Technology, USA. Antibodies to TLR4 (M300) and NF-κB p65 were purchased from Santa Cruz Biotechnology, USA. ECL® (enhanced chemiluminescence) anti-rabbit IgG, HRP (horseradish peroxidase)-linked whole antibody and ECL® Plus Western Blotting Detection System was obtained from GE Healthcare, USA.

Cell culture

Human coronary artery endothelial cells were cultured in EGM™-2 Endothelial BulletKit™ (Lonza, USA) including endothelial cell growth medium (EGM-2) and supplements with EGM-2 (5% fetal bovine serum, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, insulin-like growth-factor, ascorbic acid, epidermal growth factor, gentamicin/amphotericin-1000 and heparin). For experiments, 1×10^6/mL cells were seeded in 500 µl complete medium in 24-well plates. After growing to 80-90% confluence, the medium was changed. LPS was diluted in complete cell culture medium and added to cells. For access the time of LPS treatment, cells were exposed to LPS for 0min, 5min, 15min, 30min and 60min at 100 ng/mL. For access the concentration of LPS treatment, cells were exposed to LPS at 0 ng/mL, 1 ng/mL, 10 ng/mL,100 ng/mL and 1000 ng/mL for 30min.For access the time of atorvastatin treatment, cells were pre-treated with atorvastatin for 0h, 6h, 12h and 24h at 1µmol/l. Then, cells were exposed to LPS (100 ng/mL) for 2 h. Atorvastatin remained in culture medium for the duration for the experiment.

For access the concentration of atorvastatin treatment, cells were exposed to atorvastatin at 0 µmol/l, 0.01 µmol/l, 0.1µmol/l,1µmol/l and 10µmol/l for 12h. Then, cells were exposed to LPS (100 ng/mL) for 2 h. Atorvastatin remained in culture medium for the duration for the experiment.

For access the function of atorvastatin in inflammatory, cells were pre-treated with atorvastatin at 1µmol/l for 12h. Then, cells were exposed to LPS (100 ng/mL) for 2 h. Atorvastatin remained in culture medium for the duration for the experiment.

All the experiments were performed in triplicate, with standard deviations (SD) for each time point.

Cytokine ELISA

Levels of IL-6 and MCP-1 in cell culture supernatants were quantified by ELISA kits (R&D Systems, USA). Recombinant cytokines were used to

construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm, using a microplate reader (Bio-Rad, USA). Results were plotted against standard curves. The assays were carried out according to the protocols provided by the manufacturer.

Quantitative real-time PCR

HCAECs were seeded into six-well dishes, with a density of 5×10^4 cells per well. Cells were exposed to LPS or atorvastatin of different concentrations and stimulated for different time periods. Total cellular RNA was isolated using Trizol reagent (Invitrogen, USA). cDNAs were synthesized according to manufacturer's instructions using the Reverse Transcription kit (Promega, USA). Real-time PCR involved use of a LightCycler (Roche Applied Science, USA), following manufacturer's instruction. The transcript levels of GAPDH was quantified as an internal RNA control. Quantitative values were obtained from the threshold cycle value (Ct), the initial point when a significant increase of fluorescence was detected. Experiments were performed in triplicate, for each data point. The following primer sets were used to amplify specific cDNA fragments: GAPDH (forward: 5'-GGC TCT CCA GAA CAT CAT CC-3'; reverse:5'-TTT CTA GAC GGC AGG TCA GG-3'); TLR4 (forward:5'- AGG ATG AGG ACT GGG TAA GGA -3'; reverse: 5'- CTG GAT GAA GTG CTG GGA CA -3'); IL-6 (forward: 5'-CAT CCA TCT TTT TCA GCC ATC TTT-3'; reverse: 5'-TGA CAA ACA AAT TCG GTA CAT CCT-3'). MCP-1(forward: 5'- CAG CCA GAT GCA ATC AAT GCC-3'; reverse: 5'- TGG AAT CCT GAA CCC ACT TCT-3'). The abundance of each gene product was calculated by relative quantification, with values for the target genes, normalized with GAPDH.

Western blot analysis

Western blotting was used to detect the expression of TLR4, ICAM-1, VCAM-1, phosphorylated NF-κB p65, total NF-κB, phosphorylated p38 MAPK, total p38 MAPK, and β-actin. After treatment, HCAECs were washed three times with cold PBS, and lysed with lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Igepal, and 1× protease inhibitor cocktail) for 20 min on ice. Whole cell protein lysates were separated on 12% SDS-PAGE gels and transferred onto PVDF membranes (Invitrogen) by electro-blotting for 2 h at 60–75 V. Once dissembled, the membranes were blocked for 1 h with 5% BSA in PBST (PBS containing 0.1% Tween 20) and incubated with the appropriate primary antibodies (ICAM-1 and VCAM-1 antibody were diluted 1:200 in PBST, β-actin 1:1000, and all others 1:500) overnight at 4°C. After washing with PBST, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5000 dilution in PBST containing 5% BSA) at room temperature for 1 h. Signals were developed using ECL and exposed on X-ray films. The protein mass was compared after quantifying the intensity of protein bands using Quantity one software (Bio-Rad, Hercules, CA). Experiments were repeated three times.

Data analysis

Data were analyzed on SPSS 10.0 for Windows software. All values were expressed as means ± SD. Comparisons between groups involved use of the one-way ANOVA with Student's t test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

LPS induced TLR4 mRNA and protein expression in HCAECs

LPS has been shown previously to up-regulate TLR4 expression selectively, *in vivo* and *in vitro* (Dauphinee, Karsan, 2006) To define the optimal conditions of LPS stimulation, we examined the effects of LPS on the expression of TLR4 in HCAECs. The results showed that LPS activated TLR4 channels in HCAECs in a concentration-dependent manner (Figure 1. B, D and F). These experiments suggested the suitable concentration of LPS was 100 ng/mL.

HCAECs showed low levels of TLR4 mRNA expression in the absence of LPS stimulation. However, after stimulation with LPS (100 ng/mL), TLR4 mRNA expression rapidly increased and reached maximum expression at 30 min (Figure 1. A). In contrast, the expression of TLR4 protein showed a slower increase in expression and reached maximum expression 2 h after stimulation with LPS (100 ng/mL) in HCAECs (Figure 1. C and E).

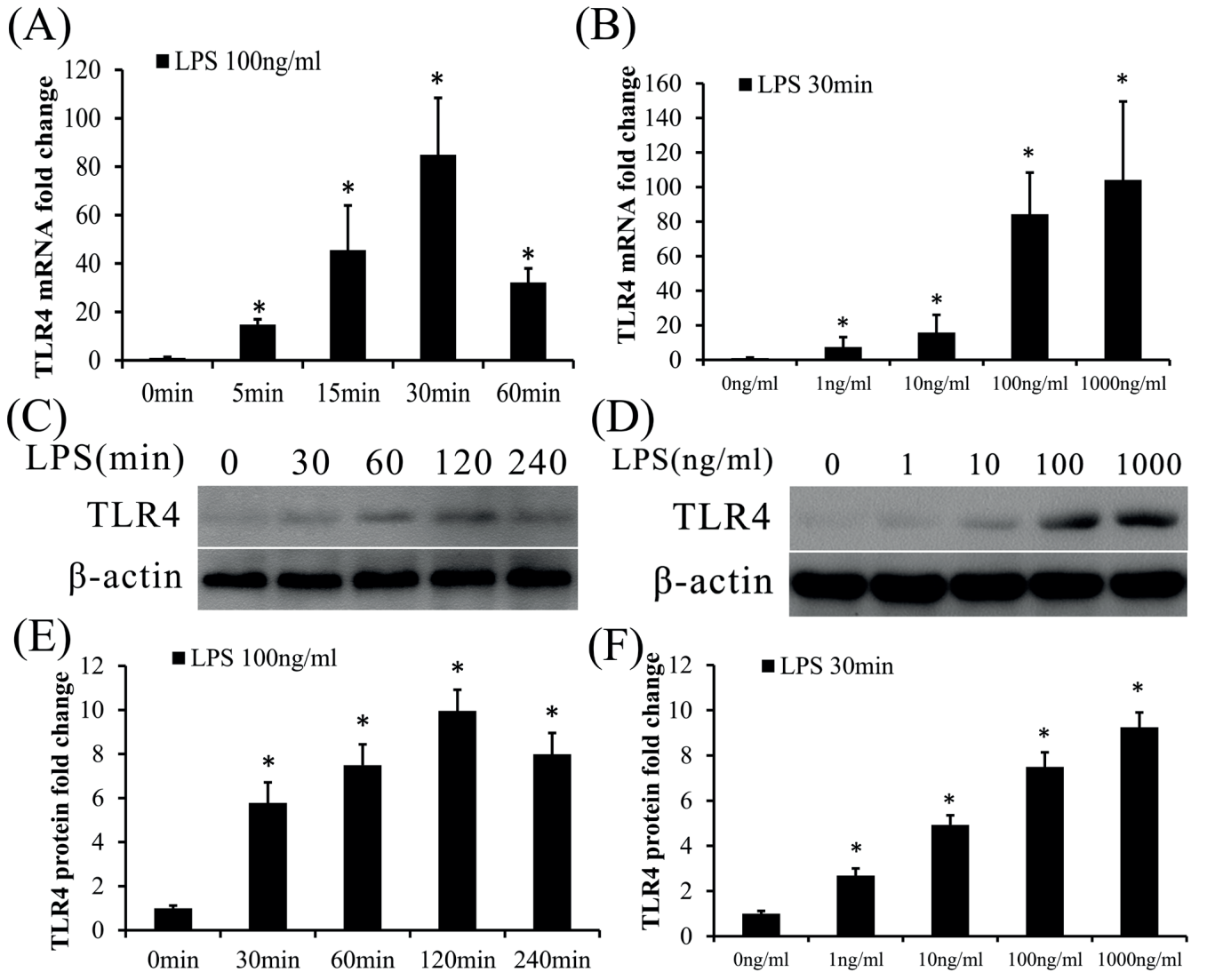

FIGURE 1 - TLR4 mRNA and protein expression in HCAECs after LPS stimulation.
(A) Time-effect TLR4 mRNA responses of LPS stimulation in HCAECs. Statistical analysis was performed on 5min, 15min, 30min and 60min versus 0min group, respectively. (B) Dose-effect TLR4 mRNA response of LPS stimulation in HCAECs. Statistical analysis was performed on 1ng/mL, 10ng/mL, 100ng/mL and 1000ng/mL versus 0ng/mL group, respectively. (C) Time-effect TLR4 protein response of LPS stimulation in HCAECs. (D) Dose-effect TLR4 protein response of LPS stimulation in HCAECs. (E) Time-effect TLR4 protein expression of TLR4 in HCAECs were quantified by using densitometry, and represented by the ratio of β-actin. Statistical analysis was performed on 5min, 15min, 30min and 60min versus 0min group, respectively. (F) Dose-effect TLR4 protein expression of TLR4 in HCAECs were quantified by using densitometry, and represented by the ratio of β-actin. Statistical analysis was performed on 1ng/mL, 10ng/mL, 100ng/mL and 1000ng/mL versus 0ng/mL group, respectively.*P<0.05 indicates significant differences.

Atorvastatin decreases LPS-induced expression of TLR4 mRNA in HCAECs

Atorvastatin has been reported to inhibit TLR4 signaling *in vivo* and *in vitro* (Methe *et al*., 2005; Wang *et al*., 2011; Fang *et al*., 2014). To clarify whether atorvastatin had the same characteristics in HCAECs, we examined the inhibitory effect of atorvastatin on TLR4 expression in these cells. Pretreatment with atorvastatin (1 μmol/l) before LPS addition (100 ng/mL) (atorvastatin+LPS) markedly reduced TLR4 mRNA level induced by LPS. In HCAECs pretreated

with atorvastatin (0.01–10.0 μmol/l) for 12 h before LPS addition (100 ng/mL), atorvastatin had a dose-dependent attenuating effect on TLR4 mRNA levels (Figure 2A). The maximal inhibition of TLR4 mRNA expression was obtained after 12 h of exposure to atorvastatin (1μmol/l) (Figure 2, A and B).

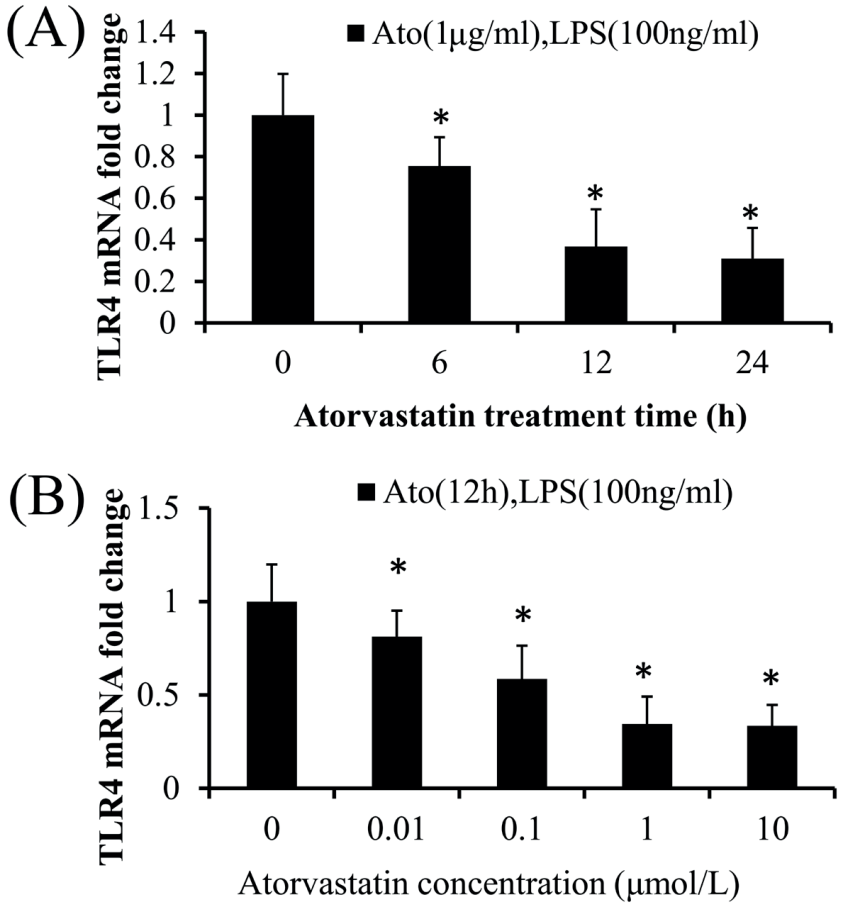

FIGURE 2 - TLR4 mRNA expression in HCAECs after atorvastatin pretreatment.
(A) Dose effect response showing TLR4 mRNA reduction treatment in HCAECs. Statistical analysis was performed on 6h, 12h, and 24h versus 0h group, respectively. (B) Time-effect response showing TLR4 mRNA reduction after atorvastatin treatment in HCAECs. Statistical analysis was performed on 0.01μmol/L, 0.1μmol/L, 1μmol/L and 10μmol/L versus 0μmol/L group, respectively.*P<0.05 indicates significant differences.

Atorvastatin inhibits LPS-induced IL-6 and MCP-1 mRNA expression and protein secretion in HCAECs

To clarify whether atorvastatin inhibit the downstream genes of TLR4 signaling in HCAECs, we examined the inhibitory effects of atorvastatin on the production of inflammatory mediators in HCAECs. For these studies, we used concentrations of 1 μM atorvastatin and pretreatments of 12 h for cell culture experiments. Using qPCR, we determined whether atorvastatin exerted inhibitory effects on the expression of the inflammatory genes, IL-6 and MCP-1 in LPS-induced HCAECs. As shown in Figure 3A and B, incubation of HCAECs with 100 ng/mL LPS for 2 h, markedly increased the mRNA expression of IL-6 and MCP-1. However, the increased IL-6 and MCP-1 mRNA levels were blunted by the additional pretreatment of atorvastatin (p <0.01).

To further confirm the inhibitory effects of atorvastatin on IL-6 and MCP-1 expression in HCAECs, these cells were pretreated with atorvastatin, then LPS was added for 2 h, and media collected for the

measurement of cytokine release using ELISA. As shown in Figure 3C and D, LPS (100 ng/mL) treatment dramatically increased the levels of all cytokines, whereas pretreatment with atorvastatin significantly reduced their levels. Atorvastatin pretreatment reduced LPS-induced IL-6 levels in the media from 1380 pg/mL to 830 pg/mL, and MCP-1 levels from 920 pg/mL to 380 pg/mL, respectively.

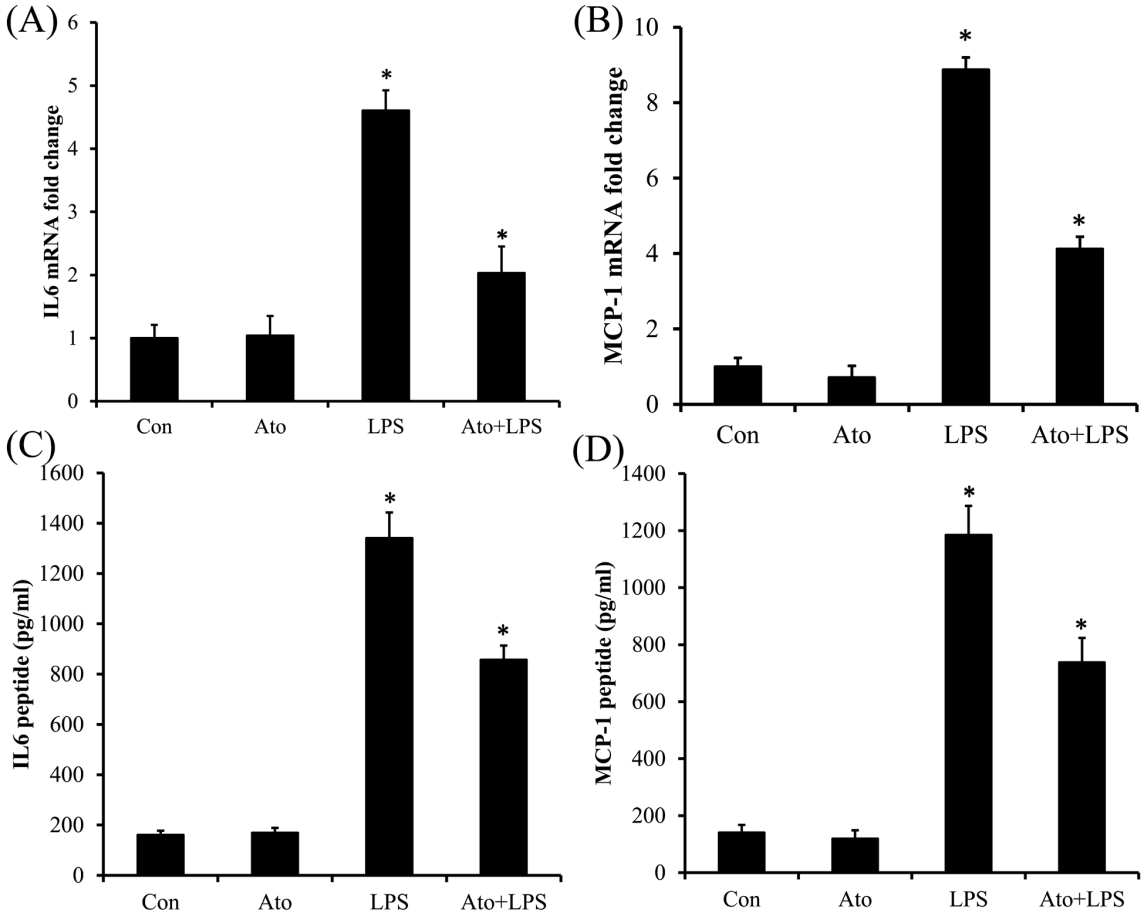

FIGURE 3 - The effects of atorvastatin on LPS-induced IL-6 and MCP-1 expression in HCAECs.

(A) IL-6 mRNA levels in HCAECs were analyzed by real-time PCR after treatment with LPS and atorvastatin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. (B) MCP-1 mRNA levels in HCAECs were analyzed by real-time PCR after treatment with LPS and atorvastatin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. (C) Levels of IL-6 peptides released in the medium were assessed by ELISA after treatment with LPS and atorvastatin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. (D) Levels of MCP-1 peptides released in the medium were assessed by ELISA after treatment with LPS and atorvastatin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. Data are expressed as mean value SE of three independent experiments. *P<0.05 indicates significant differences.

Atorvastatin suppresses LPS-induced ICAM-1 and VCAM-1 expression in HCAECs

ICAM-1 and VCAM-1 are adhesion molecules expressed by endothelial cells in response to inflammatory stimuli, and are responsible for monocyte adhesion (Iiyama *et al.*, 1999; Yoon *et al.*, 2010). The

effects of atorvastatin on the expression of ICAM-1 and VCAM-1 were tested using LPS-stimulated HCAECs by Western blot analysis (Figure 4). The results demonstrated that LPS (100 ng/mL) significantly increased ICAM-1 and VCAM-1 expression in HCAECs by 3.5- and 5.2-fold, respectively (Figure4. A and B). However, after cells were pretreated with atorvastatin (1 μM/mL) for 12 h, ICAM-1 and VCAM-1 levels induced by LPS were markedly reduced by 48.2 and 61.7%, respectively (Figure4 A and B).

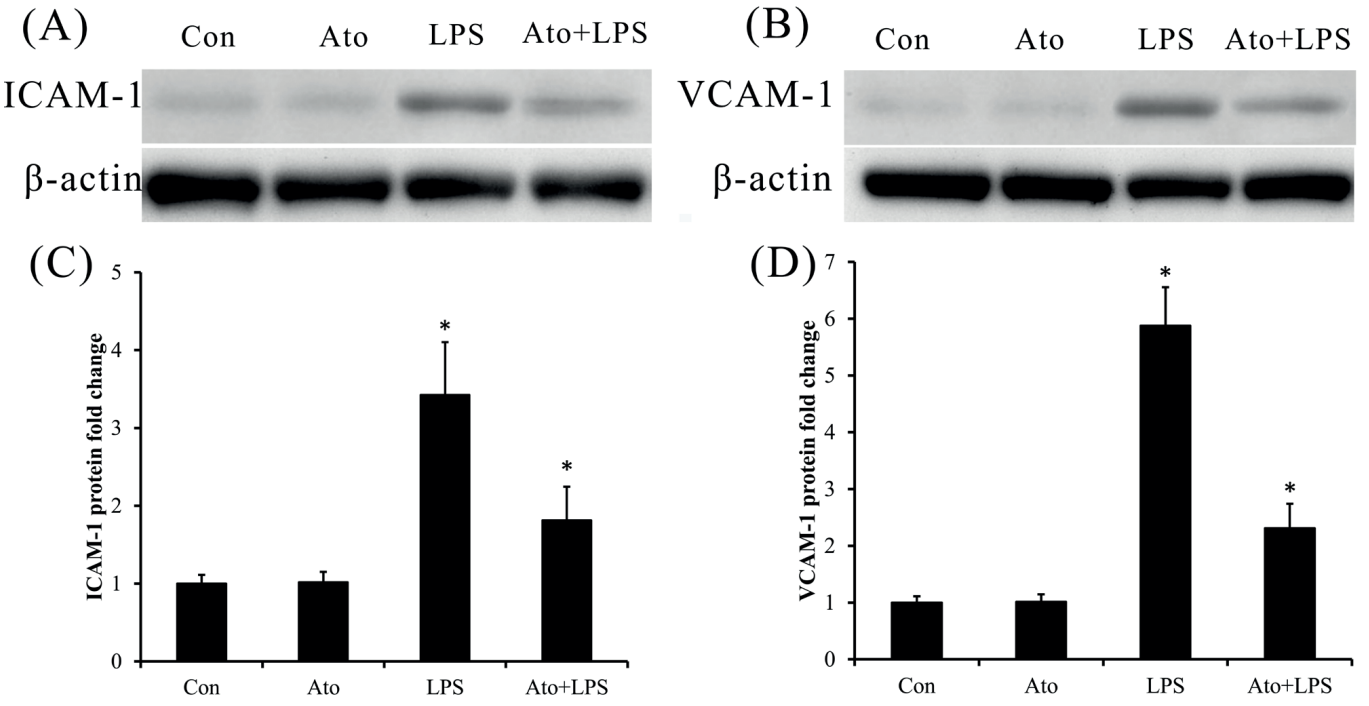

FIGURE 4 - The effects of atorvastatin on LPS-induced ICAM-1 and VCAM-1 expression in HCAECs.
(A) Protein expression of ICAM-1 in HCAECs was assessed by Western blotting after treatment with LPS and atorvastatin. (B) Protein expression of VCAM-1 in HCAECs was assessed by Western blotting after treatment with LPS and atorvastatin. (C) Protein expression of ICAM-1 in HCAECs was quantified using densitometry, and normalized to β-actin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. (D) Protein expression of VCAM-1 in HCAECs was quantified using densitometry, and normalized to β-actin. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively.Data are expressed as mean values SE of three independent experiments. *P<0.05 indicates significant differences.

Atorvastatin inhibits LPS-induced phosphorylation of NF-κB and p38 MAPK in HCAECs

We next examined the effects of atorvastatin on the NF-κB signaling pathway, which is involved in the regulation of downstream pro-inflammatory gene expression in HCAECs (Dauphinee, Karsan, 2006). The results in Figure 5A showed that LPS (100 ng/mL) treatment for 2 h induced a rapid increase in p- NF-κB protein levels in HCAECs. Pretreatment with atorvastatin at concentrations of 1 μM, reduced the level of p- NF-κB to 61.5% that of the LPS-only group. Some studies have reported the activation of the phosphatidylinositol p38 MAPK pathway and its contribution to the up-regulation of inflammatory cytokines in activated endothelial cells (Griendling *et al.*, 2000; Hsu, Wen, 2002). Thus, we also analyzed the effects of atorvastatin on LPS-induced p38 MAPK activation in HCAECs. As shown in Figure 5B, levels of p- p38 MAPK in HCAECs was rapidly increased after LPS exposure for 2h. Atorvastatin pretreatment resulted in the significant inhibition of LPS-induced P38 MAPK phosphorylation, in contrast to control groups.

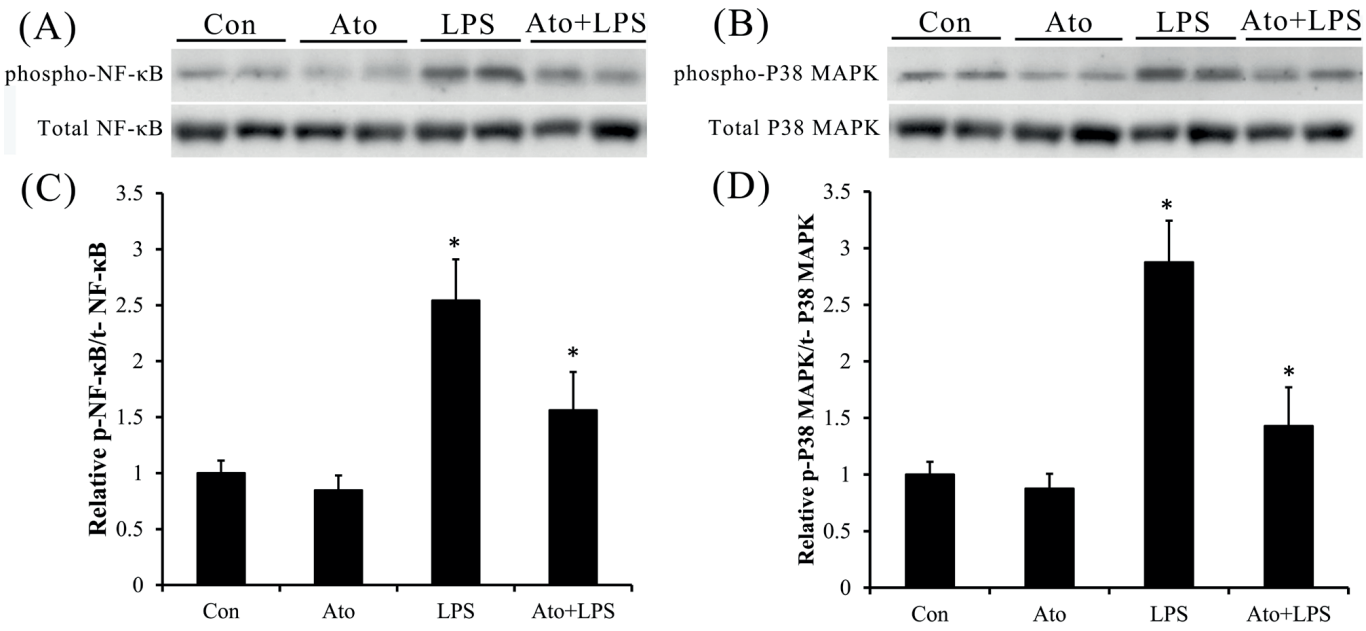

FIGURE 5 - The effects of atorvastatin on the phosphorylation of NF-κB and p38 MAPK, induced by LPS in HCAECs. (A) The phosphorylation-NF-κB was assessed by Western blotting after treatment with LPS and atorvastatin (B) The phosphorylation of p38 MAPK was assessed by Western blotting after treatment with LPS and atorvastatin. (C) The phosphorylation of NF-κB in HCAECs was quantified using densitometry and represented by the ratio of total NF-κB. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. (D) The phosphorylation of p38 MAPK in HCAECs was quantified using densitometry, and represented by the ratio of total p38 MAPK. Statistical analysis was performed on Ato, LPS and Ato+ LPS versus Con group, respectively. Data are expressed as mean values SE of three independent experiments. *P<0.05 indicates significant differences.

DISCUSSION

This study examined the anti-inflammatory effects of atorvastatin on vascular endothelial cells, using the HCAEC cell line model. Atorvastatin and potent inhibitors of cholesterol biosynthesis are widely used in the treatment of hypercholesterolemia and the prevention of atherosclerotic disease (Alagona, 2010; Sezer *et al.*, 2011). Atorvastatin exerted its anti-inflammatory effects by inhibiting TLR4 signaling, which was originally characterized as a novel anti-sepsis agent, capable of inhibiting inflammatory mediator production (Wang *et al.*, 2011). Atorvastatin inhibits TLR4 signaling by attenuating LPS-induced rapid TLR4 mRNA expression, which led to the inhibition of two distinct downstream signaling pathways: the inhibition of NF-κB translocation and the inactivation ERK phosphorylation (Fang *et al.*, 2014). Atorvastatin has also been reported to have anti-inflammatory properties in LPS-stimulated monocytes and endothelial cells (Yang *et al.*, 2012). In this study, we demonstrated that atorvastatin exerted anti-inflammatory effects in HCAECs.

TLRs are important in the innate immune response, and expression levels of these receptors reflect the sensitivity of immune cells to initiate an immune response (Abreu *et al.*, 2001; Zarember, Godowski, 2002). Several reports have indicated the regulation of TLR expression by various cytokines and molecules and have linked these observations to pathogenetic roles of TLRs in several diseases (Faure *et al.*, 2001; Xu *et al.*, 2001). Here, we demonstrate atorvastatin exerts direct regulatory effects on TLR4 expression in HCAECs that influence cellular activation. Statins reduce TLR4 surface expression on CD14 monocytes *in vivo* and *ex vivo* in a dose dependent fashion, causing down-regulation of IRAK-1 kinase activity and the reduced expression of pro-inflammatory cytokines and B7-1. Endothelial innate immune responses are key events in vascular inflammation and the development of atherosclerosis (Tousoulis *et al.*, 2006). 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) (Bjorkbacka, 2006). *In vivo* and *in vitro* studies

have also shown that high level LPS exposure triggers the activation of endothelial cells, resulting in the secretion of pro-inflammatory cytokines, which further impact on cardiovascular disease processes (Dauphinee, Karsan, 2006). Thus, up-regulation of TLR4 expression may play active roles in inflammatory disease.

The vascular inflammatory process is the result of interactions between exogenous stimuli and endothelial cells (Raetz, Whitfield, 2002; Bains *et al*., 2010). LPS, which is one of the strongest stimulators targeting the endothelium, could increase cytokine expression via a TLR4-dependent mechanism (Frost *et al*., 2002). It was reported that high level LPS exposure triggers the activation of endothelial cells, resulting in the secretion of pro-inflammatory cytokines, which further impact on cardiovascular disease processes (Dauphinee, Karsan, 2006). LPS acted as a TLR4-activating promoter, triggering pro-inflammatory responses and enhancing pro-inflammatory cytokine production (Boone *et al*., 2004). Classic pro-inflammatory cytokine genes implicated in the pathogenesis of inflammatory responses in HCAECs include IL-6, MCP-1, ICAM-1 and VCAM-1. IL-6 is an important cytokine which is expressed mainly within the endothelium of atherosclerotic plaques (Dengler *et al*., 2000; Dewberry *et al*., 2000; Shemesh *et al*., 2012). Another important cytokine produced by endothelial cells is MCP-1, which is the key player in monocyte recruitment; high MCP-1 expression levels were determined in cardiovascular disease patients (Libby, 2002; Dauphinee, Karsan, 2006). ICAM-1 and VCAM-1, produced by endothelial cells, also act as key components in inflammatory responses, are important in the recruitment of leukocytes to sites of inflammation, and are thus implicated in the pathogenesis of vascular inflammatory diseases such as atherosclerosis (Fotis *et al*., 2012). In the present study, we demonstrated that stimulation with LPS (100 ng/mL) increased IL-6, MCP-1, ICAM-1 and VCAM-1 expression in HCAECs, while pretreatment with atorvastatin suppressed LPS-induced expression of IL-6, MCP-1, ICAM-1 and VCAM-1 in these cells.

Previous studies have implicated the activation of MAPKs and NF-κB in mediating the effects of specific TLRs in various cell types (Wang *et al*., 2010; Wang *et al*., 2011). 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 p38 MAPK, and NF-κB. Our results showed that LPS up-regulated TLR4 and elicited activation of the MAPK and NF-κB signaling cascade in HCAECs, which was consistent with previous studies (Bachar *et al*., 2004). p38 MARK actives the immune response by phosphorylate several cellular targets, including cytosolic phospholipase A2, the microtubule-associated protein Tau, and the transcription factors ATF-1 and -2, MEF2A, Sap-1, Elk-1, NF-κB, Ets-1, and p53 (Ono, Han, 2000; Kyriakis, Avruch, 2001) (103,143).Several reports have showed that atorvastatin down-regulated the activity of p38 (Asehnoune *et al*., 2004; Nishida *et al*., 2005), whereas other studies have reported sustained p38 activation, even with atorvastatin treatment (Fukao, Koyasu, 2003). Our data demonstrated that atorvastatin exerted an inhibitory effect on LPS-induced phosphorylation of p38. Since the activation of both NF-κB and p38 MAPK can be triggered by TLR4 signaling, we believe that the effects of atorvastatin on NF-κB and p38 MAPK phosphorylation in HCAECs, are mediated by its inhibition of TLR4 signaling.

CONCLUSION

Our finding showed that atorvastatin down-regulated TLR4 mRNA expression in LPS-activated HCAECs; it effectively blocked LPS-induced expression of IL-6, MCP-1, ICAM-1, and VCAM-1 in HCAECs; and it significantly reduced LPS-induced phosphorylation of NF-κB and p38 MAPK in HCAECs. These findings suggest that atorvastatin might be suitable for development as an anti-inflammatory agent to suppress vascular inflammation and to prevent cardiovascular disease.

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