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# TAK-242 suppresses lipopolysaccharide-induced inflammation in human coronary artery endothelial cells

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TAK-242 (resatorvid), a novel small-molecule cyclohexene derivative, inhibits TLR4 signaling selectively. TAK-242 blocked the Toll-like receptor (TLR) 4-triggered inflammatory signaling by binding directly to a specific amino acid Cys747 in the intracellular domain of TLR4. The present study was designed to examine the effects of TAK-242 on vascular inflammatory responses in human coronary artery endothelial cells (HCAECs) challenged by lipopolysaccharide (LPS, a TLR4 ligand). The results show that TAK-242 attenuated the LPS-induced expression of interleukin (IL)-6,IL-8 and monocyte chemoattractant protein 1 both at the transcription and translation levels in HCAECs. LPS-induced endothelial cell adhesion molecules, intercellular adhesion molecular-1 and vascular cell adhesion molecule-1 expressions were also reduced by treatment with TAK-242. In addition, co-incubation with TAK-242 did not effect the expression of TLR4 in LPS-activated HCAECs. Furthermore, TAK-242 efficiently suppressed LPS-induced phosphorylation of nuclear factor κB (NF-κB) and IL-1 associated kinase-1 (IRAK-1) in HCAECs. These findings show that TAK-242 can suppress endothelial cell inflammation, suggesting that TAK-242 might be suitable for development as a therapeutic agent for inflammatory cardiovascular disease.

## 1. Introduction

Vascular local inflammation plays an important role in the development and progress of atherosclerosis (Kinlay and Ganz 1997; Simon et al. 1999; Akira et al. 2006). Endothelial cells are important in immune and inflammatory responses (Medzhitov and Janeway 2000; Takeda et al. 2003), and inflammatory activation of the endothelial cells is a critical step in the development of atherosclerosis (Simon et al. 1999; Akira et al. 2006).

Toll-like receptor-4 (TLR4) is a member of the TLR family of pathogen pattern recognition receptors that recognize bacterial and viral products, and other pathogens in the induction of innate immune and inflammatory responses (Lee et al. 2001, 2003). LPS (lipopolysaccharide), the major portion of the outer membrane of Gram-negative bacteria, is a ligand and potent agonist of TLR4 (Wiedermann et al. 1999). LPS (lipid A) is sufficient to activate TLR4 and downstream signaling pathways (Lee et al. 2001, 2003). Endothelial cells, upon LPS stimulation, initiate the over-production of inflammatory cytokines including interleukin (IL)-6, IL-8, chemokines such as MCP-1, and adhesion molecules such as intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Yamagami et al. 2003; Dauphinee and Karsan 2006). Accumulating evidence has implicated the activation or suppression of TLR4 in the development and progression of various inflammatory diseases (Schroder and Schumann 2005; Gribar et al. 2008). Thus, TLR4 is an excellent therapeutic target for the treatment of inflammatory diseases.

TAK-242, a cyclohexene derivative, is a novel small-molecule compound that selectively inhibits TLR4 signaling (Ii et al. 2006; Kawamoto et al. 2008). TLR4 is indeed the target protein of TAK-242 and that TAK-242 binds to the TIR domain of TLR4 *via* Cys747 (Takashima et al. 2009). TLR signaling is mediated through the TIR domain, the oligomerization of which initiates the recruitment of TIR domain-containing adaptor proteins. TAK-242 inhibits TLR4 signaling by disrupting the interactions of TLR4 with its adaptor molecules (Matsunaga et al. 2011). HCAECs, an endothelial cell line derived from human coronary artery endothelial cells, are commonly used for *in vitro* experimental models of vascular endothelial cells. However, the roles and mechanisms of TAK-242 in HCAECs inflammation remain unknown.

In this study, we investigated the effects of TAK-242 on the expression of LPS-induced inflammatory genes (IL-6, IL-8 and MCP-1) and adhesion molecules such as ICAM-1 and VCAM-1 in HCAECs. and the LPS-induced phosphorylation of signaling molecules NF-κB and IRAK-1 were also analyzed.

## 2. Investigations and results

### *2.1. TAK-242 decreases LPS-induced IL-6, IL-8 and MCP-1 mRNA expression and protein secretion in HCAECs*

TAK-242 has been shown previously to inhibit TLR4 signaling selectively *in vivo* and *in vitro* (Ii et al. 2006; Kawamoto et al. 2008). To clarify whether TAK-242 have the same characteristics in HCAECs, we examined the inhibitory effect of TAK-242 on the production of inflammatory mediators in HCAECs. Therefore we chose to use concentrations of 1 μM TAK-242 for cell culture experiments throughout these studies. We determined whether TAK-242 had inhibitory effects on the expression of inflammatory genes IL-6, IL-8 and MCP-1 in LPS-induced HCVECs by qPCR. As shown in Fig. 1, incubation of HCVECs with 100 ng/ml of LPS for 1 h markedly increased the mRNA expression of IL-6, IL-8 and MCP-1. However, the increased IL-6, IL-8 and MCP-1 mRNA levels were all blunted by the additional treatment of TAK-242 ($p < 0.01$).

To further confirm the inhibitory effects of TAK-242 on IL-6,IL-8 and MCP-1 expression in HCAECs, HCAECs were treated with TAK-242 and LPS for 2 h, and media were collected for measurement of cytokine concentrations using ELISA. As shown in Fig. 1D, E and F, LPS (100 ng/ml) treatment dramatically increased the levels of all cytokines, whereas co-treatment with TAK-242 significantly reduced their levels. TAK-242 co-treatment reduced LPS-induced IL-6 levels in the media from 1340 pg/ml to 160 pg/ml, IL-8 levels from 1453 pg/ml to 340 pg/ml and MCP-1 levels from 1180 pg/ml to 140 pg/ml, respectively.

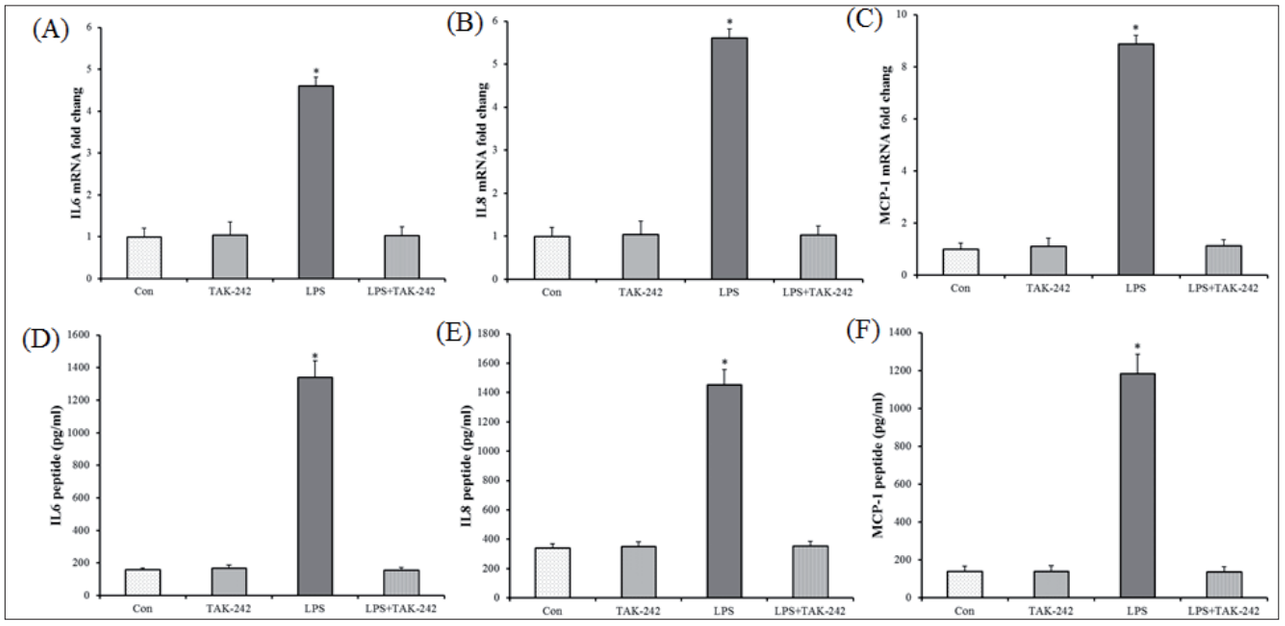


Fig. 1: Effect of TAK-242 on LPS-induced IL-6, IL-8 and MCP-1 expression in HCAECs. (A) IL-6 mRNA levels in hCAEC were analyzed with real-time PCR after treatment by LPS and TAK-242. (B) IL-8 mRNA levels in hCAEC were analyzed with real-time PCR after treatment by LPS and TAK-242. (C) MCP-1 mRNA levels in hCAEC were analyzed with real-time PCR after treatment by LPS and TAK-242. (D) Levels of IL-6 peptides in medium were assessed by ELISA after treatment by LPS and TAK-242. (E) Levels of IL-8 peptides in medium were assessed by ELISA after treatment by LPS and TAK-242.(F) Levels of MCP-1 peptides in medium were assessed by ELISA after treatment by LPS and TAK-242. Data are expressed as mean values SE of three independent experiments, * $p < 0.05$ was considered to be a statistically significant difference.

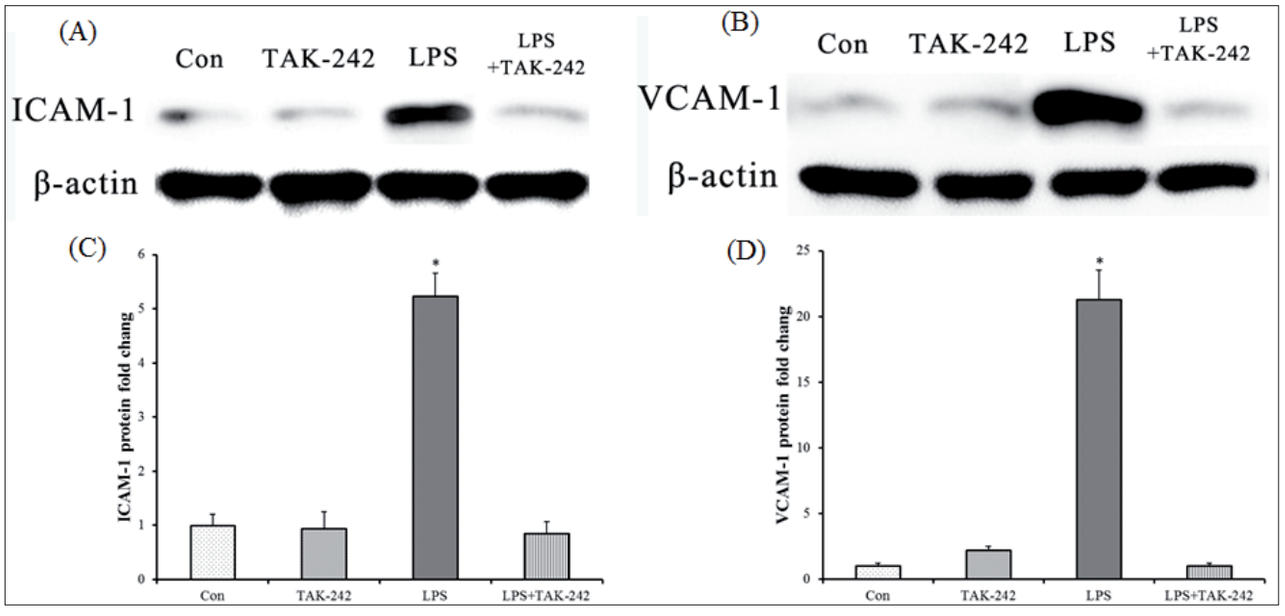


Fig. 2: Effects of TAK-242 on the LPS-induced ICAM-1 and VCAM-1 expression in HCAECs . (A) Protein expression of ICAM-1 in hCAECs was assessed by western blot after treatment by LPS and TAK-242. (B) Protein expression of VCAM-1 in hCAECs was assessed by western blot after treatment by LPS and TAK-242. (C) Protein expression of ICAM-1 in hCAECs was quantified by using densitometry, and represented by the ratio of β-actin. (D) Protein expression of VCAM-1 in hCAECs was quantified by using densitometry, and represented by the ratio of β-actin. Data are expressed as mean values SE of three independent experiments, * $p < 0.05$ was considered to be a statistically significant difference.

### *2.2.* TAK-242 suppresses LPS-induced the ICAM-1 and VCAM-1 protein expression in HCAECs

ICAM-1 and VCAM-1 are two of several 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 TAK-242 on the expression of ICAM-1 and VCAM-1 were tested using LPS-stimulated HCAEs by Western blot analysis (Fig. 2). The results demonstrated that LPS (100 ng/ml) significantly increased ICAM-1 and VCAM-1 expression in HCAEs by 5.3- and 23-fold, respectively. However, after the cells were co-treated with TAK-242 (1 μM), the ICAM-1 and VCAM-1 levels induced by LPS were markedly reduced to normal level.

### *2.3. Both TAK-242 and LPS do not alert TLR4 mRNA and protein level in HCAECs*

TAK-242 binds directly to TLR4 *via* Cys747 in the TIR domain of TLR4 and inhibits TLR4 signals (Takashima et al. 2009). To clarify whether TAK-242 influences the expression of TLR4 in HCAECs,

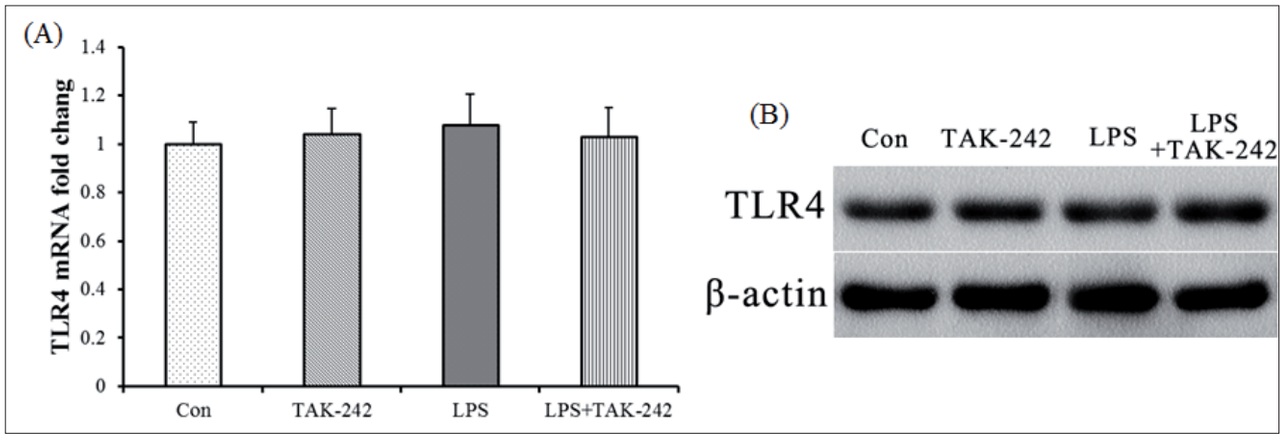


Fig. 3: Effects of TAK-242 on the LPS-induced TLR4 expression in HCAECs. (A) TLR4 mRNA levels in hCAEC were analyzed with real-time PCR after treatment by LPS and TAK-242. (B) Levels of TLR4 were assessed by western blot after treatment by LPS and TAK-242. Data are expressed as mean values SE of three independent experiments, * p <0.05 was considered to be a statistically significant difference.

we examined the expression of TLR4 in HCAECs. Figure 3 shows that both mRNA and protein of TLR4 were not changed after treatment by LPS and TAK-242 in contrast to the control group. These results suggest that the inhibitory effect of TAK-242 may result from the direct action of TAK-242 on TLR4 without requiring the reduction of TLR4 gene expression and protein synthesis in HCAECs.

### *2.4. TAK-242 inhibits LPS-induced IRAK-1 and NF-κB phosphorylation in HCAECs*

Next, we examined the effects of TAK-242 on the NF-κB signaling pathway, which is involved in the regulation of downstream proinflammatory gene expression in HCAECs (Dauphinee and Karsan 2006). The results in Fig. 4A show that LPS (100 ng/mL) treatment for 1 h induced a rapid increase in p- NF-κB protein levels in HCAECs. Co-treatment with TAK-242 at concentrations of 1 μM reduced the level of p-NF-κB to 29.3 % that of the LPS-only group. Several studies have reported the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and its contribution to the upregulated production of inflammatory cytokines in activated endothelial cells (Lye et al. 2004). Thus, we also analyzed the effects of TAK-242 on LPS-induced IRAK-1activation in HCAECs. As shown in Fig. 4B, the level of p- IRAK-1 in HCAECs was rapidly increased after LPS exposure for 1h. TAK-242 co-treatment resulted in the significant inhibition of LPS-induced IRAK-1 phosphorylation in contrast to control group.

## 3. Discussion

This study aimed to examine the anti-inflammatory effects of TAK-242 on vascular endothelial cells, using the HCAEC cell line as a model. TAK- 242 (resatorvid), a cyclohexene derivative, is a small-molecule inhibitor of TLR4 signaling, which was originally characterized as a novel anti-sepsis agent capable of inhibiting inflammatory mediator production (Takashima et al. 2009). TAK-242 inhibits TLR4 signaling by disrupting the interactions of TLR4 with its adaptor molecules and is the only small molecule reported to regulate protein-protein interactions between TLR4 and its adaptor molecules (Matsunaga et al. 2011). It has been reported that the anti-inflammatory properties of TAK-242 in LPS-stimulated monkey fibroblast, mouse myoblasts and human HEK293 cell

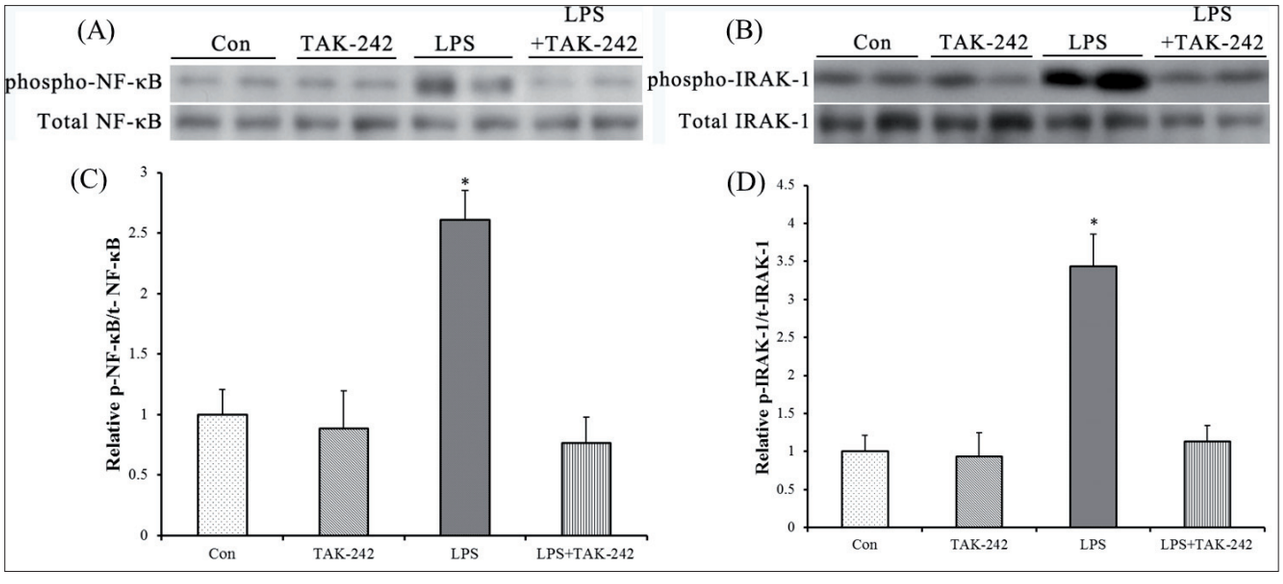


Fig. 3: Effects of TAK-242 on phosphorylation of NF-κB and IRAK-1 induced by LPS in HCAECs. (A) Levels of phosphorylation-NF-κB were assessed by western blot after treatment by LPS and TAK-242; (B) Levels of phosphorylation-IRAK-1 were assessed by western blot after treatment by LPS and TAK-242. (C) Levels of phosphorylation-NF-κB in hCAECs were quantified by using densitometry, and represented by the ratio of total NF-κB. (D) Levels of phosphorylation-IRAK-1 in hCAECs were quantified by using densitometry, and represented by the ratio of total IRAK-1.Data are expressed as mean values SE of three independent experiments, * p <0.05 was considered to be a statistically significant difference.

s(Takashima et al. 2009; Matsunaga et al. 2011; Hussey et al. 2012). In this study, we demonstrated that TAK-242 also exerted anti-inflammatory effects on human vascular endothelial cells.

Endothelial innate immune responses are key events in vascular inflammation and the development of atherosclerosis (Tousoulis et al. 2006). The vascular inflammatory process is the result of the interaction between exogenous stimuli and endothelial cells (Raetz and Whitfield 2002; Bains et al. 2010). LPS, which is one of the strongest stimulators that target the endothelium, could increase cytokine expression *via* a TLR4-dependent mechanism (Frost et al. 2002). *In vivo* and *in vitro* studies have also shown that high-level LPS exposure can trigger activation of endothelial cells, resulting in the secretion of pro-inflammatory cytokines, with further impact on cardiovascular disease processes (Dauphinee and Karsan 2006). LPS could act as TLR4-activating promoters, trigger pro-inflammatory responses and enhance pro-inflammatory cytokine production (Boone et al. 2004). Classic pro-inflammatory cytokine genes involved in the pathogenesis of inflammatory responses in HCAECs include IL-6, IL-8 ,MCP-1, ICAM-1 and VCAM-1.IL-6 and IL-8 are important cytokine which is expressed mainly within the endothelium of atherosclerotic plaques (Dengler et al. 2000; Dewberry et al. 2000; Shemesh et al. 2012). An important cytokine produced by ECs is MCP-1, which is the key player in monocyte recruitment, and high MCP-1 expression levels were determined in cases of cardiovascular disease (Libby 2002; Dauphinee and Karsan 2006). ICAM-1 and VCAM-1, produced by ECs, also act as key components in the inflammatory response, as well as in the recruitment of leukocytes to the 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, IL-8, MCP-1, ICAM-1 and VCAM-1 expression in HCAECs, while co-treatment with TAK-242 was able to suppress LPS-induced expression of all five.

TAK-242 can inhibit TLR4 signaling *via* direct binding to a specific amino acid in ICD of TLR4 and has no binding affinity to other characterized TLRs (Takashima et al. 2009). Therefore, the reduction of TLR4 will also inhibit LPS-induced IL-6, IL-8, MCP-1, ICAM-1 and VCAM-1 expression, so we detected the expression of TLR4 in HCAECs. The results show that co-incubation of HCAECs with TAK-242 has no effect on the expression of TLR4, both on mRNA and protein levels. It has been reported that the NF-κB signaling pathway is involved in the regulation of ICAM-1 and VCAM-1 expression (Swantek et al. 2000; Lye et al. 2004; Wertz et al. 2004; Cusson-Hermance et al. 2005). In addition, the activation of NF-κB has been shown to be associated with the expression of pro-inflammatory mediators which can trigger vascular inflammation (Swantek et al. 2000; Lye et al. 2004; Wertz et al. 2004; Cusson-Hermance et al. 2005). Thus, we investigated whether TAK-242 modulates NF-κB and IRAK-1 phosphorylation, which might contribute to the reduction of VCAM-1, ICAM-1, MCP-1 and IL-1β expression in TAK-242 treated HCAECs. Our results showed that the phosphorylation of NF-κB and IRAK-1 induced by LPS was inhibited by TAK-242 in HCAECs. Since the activation of both NF-κB and IRAK-1can be triggered by TLR4 signaling, we believe that the effects of TAK-242 on NF-κB and IRAK-1 phosphorylation in HCAECs are mediated by its inhibition of TLR4 signaling.

In conclusion, we found that TAK-242 effectively blocked LPS-induced expression of IL-6, IL-8, MCP-1, ICAM-1, and VCAM-1 in HCAECs; had no effect on the expression of TLR4 in LPS-activated HCAECs; and significantly reduced LPS-induced phosphorylation of NF-κB and IRAK-1 in HCAECs. These findings suggest that TAK-242 might be suitable for development as an anti-inflammatory agent to suppress vascular inflammation and to prevent and treat cardiovascular diseases.

## 4. Experimental

### *4.1. Chemicals and reagents*

EBM-2 (endothelial cell growth medium-2),was obtained from from Lonza. FBS (fetal bovine serum), gentamicin/amphotericin-1000, and trypsin/EDTA, PBS and Hepes buffer solution were obtained from Gibco. LPS from *Escherichia coli* J5 (L5014), 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 noted, were from Sigma. Antibodies to ICAM-1,VCAM-1, phosphorylated IRAK-1 and β -actin were from Cell Signaling Technology. Antibodies to TLR4 (M300) and NF-κB p65 were from Santa Cruz Biotechnology. ECL® (enhanced chemiluminescence) anti-rabbit IgG, HRP (horseradish peroxidase)- linked whole antibody and ECL® Plus Western Blotting Detection System was obstained from GE Healthcare.

### *4.2. Preparation of TAK-242 solution*

TAK-242 [resatorvid, ethyl (6*R*)-6-[*N*-(2-chloro- 4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate], was a gift from Takeda Pharmaceuticals. Stock solutions of TAK-242 were dissolved in DMSO to a final concentration of 100 mM, and were stored at -80 °C. Prior to use, stock solutions were thawed and dissolved in EBM-2 to a final concentration of 1 μM. The 0.001 % DMSO solution in EBM-2 served as a vehicle control.

### *4.3. Cell culture*

Cells were grown in endothelial cell growth medium (EBM-2) supplemented with EGM-2 (2% 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 the experiments, cells were seeded in 500 μl complete medium in 24-well plates. After growing to confluence, medium was changed completely. LPS was diluted in complete cell culture medium and added to the cells. Cells were exposed to LPS (100 ng/ml) for 2 h (inflammatory assays). Prior to treatment with LPS, cells were pre-treated with TAK-242 (1 μM) or vehicle control for 1 h. TAK-242 remained in culture medium for the duration for the experiment.

### *4.4. Cytokine ELISA*

The amounts of IL-6, IL-8 and MCP-1 in cell culture supernatants were quantified by ELISA kits (R&D Systems, Minneapolis, MN, 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, Hercules, CA, USA). Results were plotted against the standard curve. The assays were carried out according to the protocols provided by the manufacturer.

### *4.5. RNA-isolation and real-time RT-PCR*

Confluent hCAECs were treated with LPS for 1 h. Thereafter cells were harvested with lysis buffer, and total RNA was extracted using a Qiagen RNeasy Mini Kit (Valencia, CA, USA). cDNA was prepared by reverse transcription (SuperScript III First-Strand, Invitrogen,Carlsbad, CA, USA). Real-time PCR was performed as previously reported using Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with Corbbet Cycler (Qiagen, Valencia, CA, USA). Each reaction was carried out for 45 or 50 cycles in a total volume of 15 μl (4.9 μl H2O, 7.5 μl Sybr Green Mix, 0.3 μl of each 5 mM primer, 2.0 μl cDNA). The following sets of primers 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'); IL-8 (forward:5'-CTC TTG GCA GCC TTC CTG ATT-3'; reverse: 5'-TAT GCA CTG ACA TCT AAG TTC TTT AGC A-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.

### *4.6. Western blot*

Western blot was used to detect ICAM-1, VCAM-1, phosphorylated NF-κB p65, total NF-κB, phosphorylated IRAK-1, total IRAK-1, TLR4, and beta-actin. After treatment, hCAECs were washed three times with cold PBS, and then lysed with lysis buffer (protease inhibitor cocktail and Mammalian Protein Extraction Reagent, Thermo Scientific, Waltham, MA, USA). Samples were separated on 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred onto PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% BSA in PBST (PBS containing 0.1% Tween 20), and then incubated with the appropriate primary antibodies (ICAM-1 and VCAM-1 antibody were diluted 1:200, beta-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 with PBST containing 5% BSA) at room temperature for 1 h. Bands were developed using ECL and exposed on X-ray films. Band density was analyzed using NIH ImageJ software.

### *4.7. Data analysis*

The results are represented as the mean±standard error of the mean (SEM). The data were analyzed with analysis of variance (ANOVA) tests using Statview version 5 software (SAS Institute, Inc.,Cary, NC). A Fisher's post hoc test was used to determine the means that were significantly different from the control mean. Statistical significance was assigned to p values $<0.05$.

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