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Cerivastatin Suppresses Lipopolysaccharide-Induced ICAM-1 Expression through Inhibition of Rho GTPase in BAEC

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We investigated the effect of cerivastatin on lipopolysaccharide (LPS)-induced intercellular adhesion molecule-1 (ICAM-1) expression in bovine aortic endothelial cells. Cerivastatin suppressed LPS-induced ICAM-1 mRNA expression. Cotreatment with geranylgeranylpyrophosphate reversed the effect of cerivastatin. Because Rho undergoes geranylgeranyl modification, we elucidated whether Rho is involved in LPS-induced ICAM-1 expression. Inhibition of Rho activity by *Clostridium botulinum* C3 transferase or by overexpression of RhoA T19N, a dominant-negative mutant of RhoA, decreased LPS-induced ICAM-1 expression. Although cerivastatin up-regulated endothelial nitric oxide synthase (eNOS), inhibition of nitric oxide (NO) synthesis by cotreatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) exhibited no influence on the effect of cerivastatin. The present results indicate that cerivastatin prevents LPS-induced ICAM-1 expression in endothelial cells via inhibition of Rho activity. This inhibitory effect is likely unrelated to up-regulation of eNOS.

An attachment of monocytes to endothelial cells is a key phenomenon in the pathogenesis of various diseases such as inflammation, vasculitis, and atherosclerosis (1). Adhesion molecules including selectins, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which are induced by inflammatory cytokines, lipopolysaccharide (LPS) and oxidatively modified lipids on the surface of endothelial cells, are responsible for the adherence, migration, and accumulation of monocytes (2, 3). LPS is a potent activator of cells of immune and inflammatory systems, including endothelial cells and myeloid cells, and contributes to progression of cytotoxic changes (4). Two distinct mechanisms in LPS-induced activation of endothelial cells may occur: one is direct activation by LPS and the other is indirect stimulation via cytokines released from LPS-stimulated myeloid cells (5, 6). Although the LPS-induced intracellular signaling mechanisms in monocytes or macrophages are well studied (7, 8), the intracellular signaling pathways that bring about these responses in endothelial cells are poorly understood.

Recent studies have shown that 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors have anti-atherosclerotic effects, beyond normalization of hypercholesterolemia, by directly acting on endothelial cells, macrophages and vascular smooth muscle cells (9). Some of the beneficial effects of HMG-CoA reductase inhibitors against atherogenic changes could be attributed to their ability to suppress the synthesis of isoprenoid intermediates such as geranylgeranylpyrophosphate (GGPP) or farnesylpyrophosphate (FPP) rather than their ability to interrupt cholesterol synthesis (10). These effects of HMG-CoA reductase inhibitors beyond normalization of hypercholesterolemia are thought to operate also *in vivo,* and may contribute to clinical outcome that HMG-CoA reductase inhibitors decrease the incidence of ischemic strokes and myocardial infarction in atherosclerotic patients. Furthermore, it was also shown that HMG-CoA reductase inhibitors reduce cardiovascular events in patients without hypercholesterolemia (11).

Atherosclerosis has become to be recognized as an inflammatory disease (1). The cellular interactions in atherogenesis are not fundamentally different from those in other inflammatory diseases. It has been shown that HMG-CoA reductase inhibitors can prevent or restore changes in endothelial cells, macrophages, and vascular smooth muscle cells associated with atherosclerosis by altering intercellular signal-

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ings or cellular interactions. We hypothesized that HMG-CoA reductase inhibitors may exert beneficial potency towards inflammatory cellular responses in general by modulating expression of adhesion molecules via inhibition of intracellular signaling pathways in endothelial cells.

In this study, we demonstrate that LPS-induced ICAM-1 expression pathway is suppressed by cerivastatin, an HMG-CoA reductase inhibitor. This suppressive effect of cerivastatin is independent of up-regulation of endothelial nitric oxide synthase (eNOS), but reversed by cotreatment with GGPP. *Clostridium botulinum* C3 transferase or overexpression of a dominant-negative mutant of RhoA, prevents LPS-induced ICAM-1 expression. These results indicate that Rho is involved in LPS-induced ICAM-1 expression pathway, and that cerivastatin down-regulates this pathway by blocking geranylgeranylation of Rho.

MATERIALS AND METHODS

Materials. All standard culture reagents were obtained from Gibco BRL (Grand Island, NY). Cerivastatin was kindly provided from Bayer. LPS, GGPP, and FPP were from Sigma Chemical Co. (St. Louis, MO). Clostridium botulinum C3 transferase was from Wako Pure Chemicals (Osaka, Japan). [α-^{32}P]dCTP was from Amersham International.

Cell culture. Bovine aortic endothelial cells (BAECs) were harvested from bovine thoracic aorta as described previously (12) and identified by their typical cobblestone appearance and positive immunofluorescence for factor VIII. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 10 μg/ml sulbenicillin and 15 μg/ml gentamicin at 37°C in a 95% air and 5% CO_2 atmosphere. Cells were used between passages 5 and 13.

Overexpression of a mutant of RhoA. Transient transfection was carried out using replication-defective recombinant adenoviral vectors prepared as described previously (13). Briefly, a dominant-negative mutant of RhoA (Thr 19 to Asn, RhoA T19N), which was generously provided by Dr. Y. Takai (Osaka University, Osaka, Japan), was placed into pAdex1CAwt, a cassette cosmid vector (kindly provided by Dr. I. Saito (University of Tokyo) under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter (pAdex RhoA T19N). A recombinant adenovirus was constructed by *in vitro* homologous recombination in 293 cells using pAdex RhoA T19N and the adenovirus DNA-terminal protein complex. Cells were grown on 6 cm culture dishes. After reaching confluence, cells were infected with recombinant adenovirus expressing RhoA T19N, a dominant-negative mutant of RhoA, or LacZ diluted in DMEM with 5% fetal calf serum at a multiplicity of infection (MOI) of 30 particles/cell and incubated for 24 h. The viral suspension was removed and cells were cultured in the serum depleted medium for 24 h.

cDNA probes of bovine ICAM-1 and bovine eNOS. Total RNA (1 μg), extracted from LPS-stimulated BAEC, was reverse transcripted with Oligo dT (Promega) and M-MLV reverse transcriptase (Promega) at 37°C for 3 h. One μg of the reverse-transcripted material was amplified with *Taq* DNA polymerase (Takara) using a primer pair specific to bovine ICAM-1 cDNA (5′-gtgaattccatggcaccaatttctct-3′, 3′-gtgaattcacttcacgggtgactctg-5′) (14). PCR product was digested with EcoRI and ligated into EcoRI-site of pBluescript. The cDNA insert was confirmed by sequenced by the dideoxy termination method. ICAM-1 cDNA probe was obtained from the amplified pBluescript containing ICAM-1 cDNA.

Full-length of eNOS cDNA was digested with SacI. cDNA extracted from fragments of 1.1 kbp was used as eNOS cDNA probe for Northern blot analysis (15).

Northern blot analysis. Total RNAs were extracted from cells with ISOGEN (Wako). Equal amounts of total RNA (15 μg) were size-fractionated on 1% agarose gel containing 5% formaldehyde, and RNAs were transferred to a Hybond-N membrane (Amersham) by blotting using 10× SSC buffer. Blots were hybridized at 42°C in buffer containing 50% formamide, 5× SSC, 1× Denhardt's solution, and 0.5% SDS. cDNA probes of bovine ICAM-1 or bovine eNOS were labeled by random priming (Amersham) using [α-^{32}P]dCTP. After washed twice at 55°C in 0.2% SSC and 0.1% SDS for 30 min, filters were exposed to imaging plates for 45 min, and processed to the imaging analysis. RNA loading was determined by ethidium bromide staining of 18S and 28S ribosomal RNA.

RESULTS

Cerivastatin Suppressed LPS-Induced ICAM-1 mRNA Expression

Northern blot analysis showed that LPS (50 ng/ml) induced a time-dependent increase up to 24 h in ICAM-1 mRNA expression in BAECs (data not shown). Pretreatment of BAECs with cerivastatin (0.1–10 μM) for 16 h prior to stimulation with LPS (50 ng/ml) resulted in a concentration-dependent suppression of LPS-induced ICAM-1 expression (Fig. 1A). In contrast, tumor necrosis factor-α (TNF-α) (100–500 U/ml)-induced ICAM-1 mRNA expression reached the maximal level at 3–6 h and then decreased gradually (data not shown). Pretreatment with cerivastatin had no suppressive effect on TNF-α-induced ICAM-1 expression (Fig. 1B). Thus cerivastatin suppressed LPS-induced ICAM-1 mRNA expression in BAECs, whereas this agent failed to modify TNF-α-induced ICAM-1 mRNA expression.

Effect of Cerivastatin on eNOS mRNA Expression

Recent reports demonstrated that HMG-CoA reductase inhibitors up-regulate eNOS expression and nitric oxide (NO) production (16, 17), and NO is revealed to inhibit ICAM-1 expression in endothelial cells (18). We examined whether the suppression of LPS-induced ICAM-1 mRNA expression by cerivastatin is caused by NO production via up-regulation of eNOS. To this end, we first assessed the effect of cerivastatin on eNOS mRNA expression levels. Treatment with LPS alone only minimumly modified eNOS mRNA expression. Cerivastatin induced up-regulation of eNOS mRNA expression in LPS-treated endothelial cells, which was inhibited by cotreatment with isoprenoid intermediates, geranylgeranylpyrophosphate (GGPP) at a concentration of 10 μM, but not farnesylpyrophosphate (FPP) (1–10 μM) (Fig. 2A). This finding is in accordance with the report of Laufs *et al.*, where they demonstrated that HMG-CoA reductase inhibitors up-regulate eNOS mRNA expression by increasing its stability via blocking of geranylgeranylation of Rho (19).

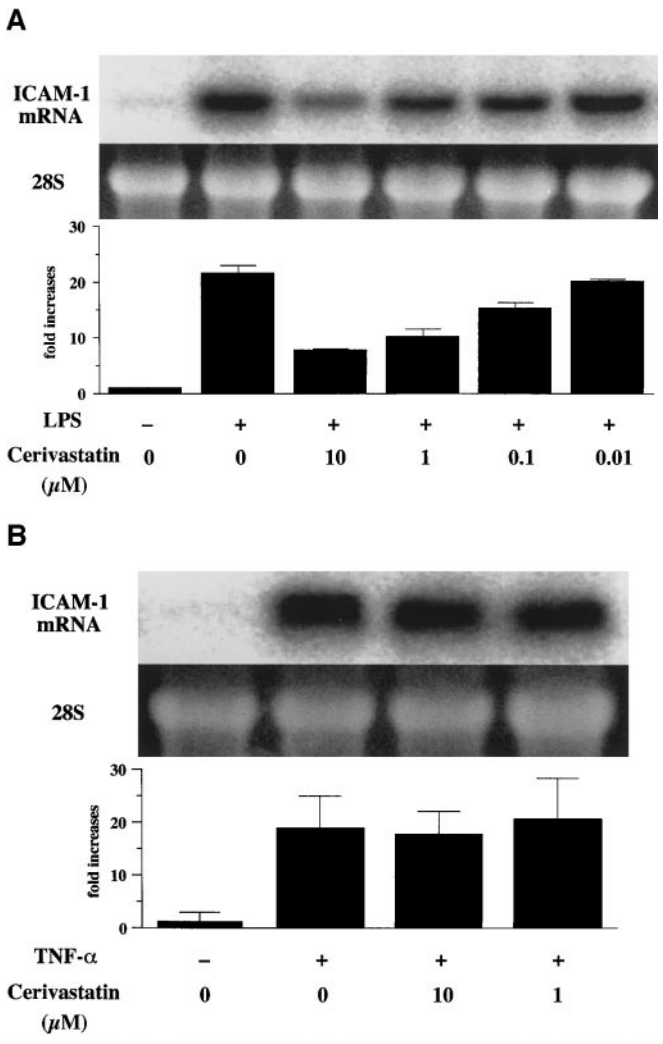

FIG. 1. Cerivastatin suppressed LPS-induced ICAM-1 expression. After pretreatment with cerivastatin (0.01–10 μM) for 16 h, confluent BAECs were treated with LPS (50 ng/ml) for 10 h (A) or TNF-α (100 U/ml) for 3 h (B), and subsequently total RNAs were extracted. Northern blot analysis was performed using cDNA probes of bovine ICAM-1. Representative results are shown in the upper panel. Fold increases of ICAM-1 mRNA expression compared with basal expression are shown in the lower panel. The values are means ± SEM of at least 3 independent experiments.

Next we examined whether the inhibitory effect of cerivastatin on LPS-induced ICAM-1 mRNA expression could be mediated by NO. We found that inhibition of NO synthesis by pre-treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) (1 mM) exhibited no influence on the effects of cerivastatin (Fig. 2B).

Effect of Isoprenoids on LPS-Induced ICAM-1 Expression

Then we examined the direct effect of cerivastatin on LPS-induced ICAM-1 mRNA expression. BAECs, pretreated with cerivastatin (10 μM), were stimulated with LPS (50 ng/ml) in the presence of GGPP or FPP. Cotreatment with FPP (1–10 μM) did not reverse the suppressive effect of cerivastatin on LPS-induced ICAM-1 expression. In contrast, cotreatment with GGPP at a concentration of 10 μM, but not 1 μM, reversed the suppressive effect of cerivastatin (Fig. 3A). These findings indicate that LPS-induced ICAM-1 expression pathway is positively regulated by geranylgeranyl synthesis, and that cerivastatin suppresses this pathway by blocking geranylgeranyl synthesis. In addition, treatment with GGPP or FPP did not affect basal levels of ICAM-1 expression or LPS-induced ICAM-1 expression.

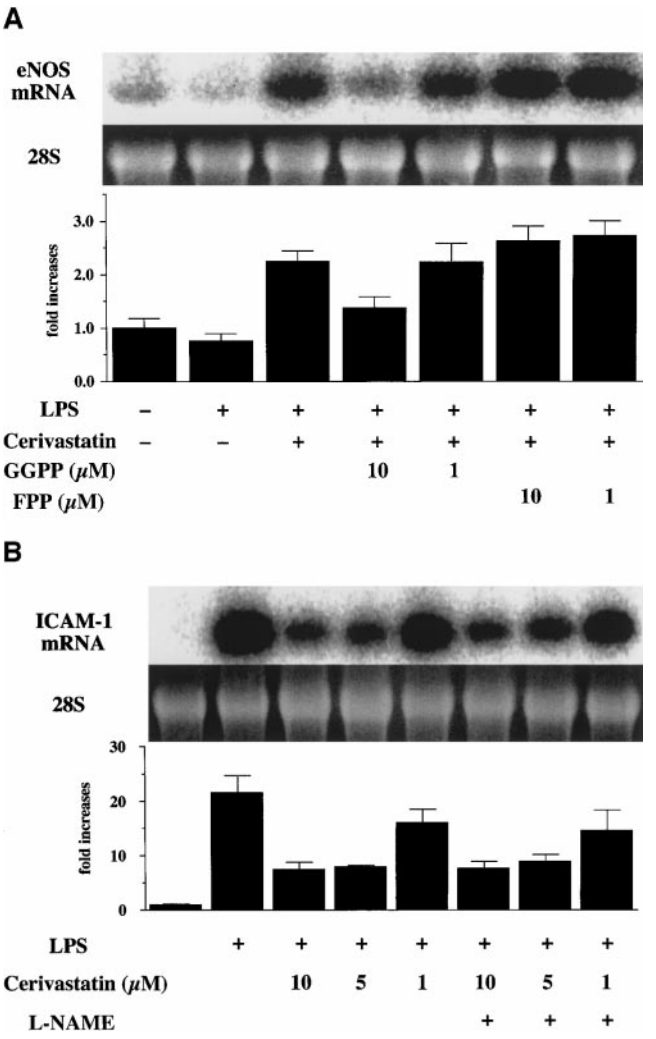

FIG. 2. L-NAME has no effects on suppression of ICAM-1 expression. (A) Confluent BAECs were pretreated with cerivastatin (10 μM) in the presence or absence of GGPP or FPP for 16 h and subsequently stimulated with LPS for 10 h. (B) BAECs were pretreated with cerivastatin (0.1–10 μM) for 16 h and subsequently stimulated with LPS in the presence or absence of L-NAME (1 mM), added 1 h prior to stimulation with LPS. Northern blot analysis was performed using cDNA probes of bovine eNOS (A) or ICAM-1 (B). Representative results are shown in the upper panel. Fold increases of ICAM-1 mRNA expression compared with basal expression are shown in the lower panel. The values are means ± SEM of at least 3 independent experiments.

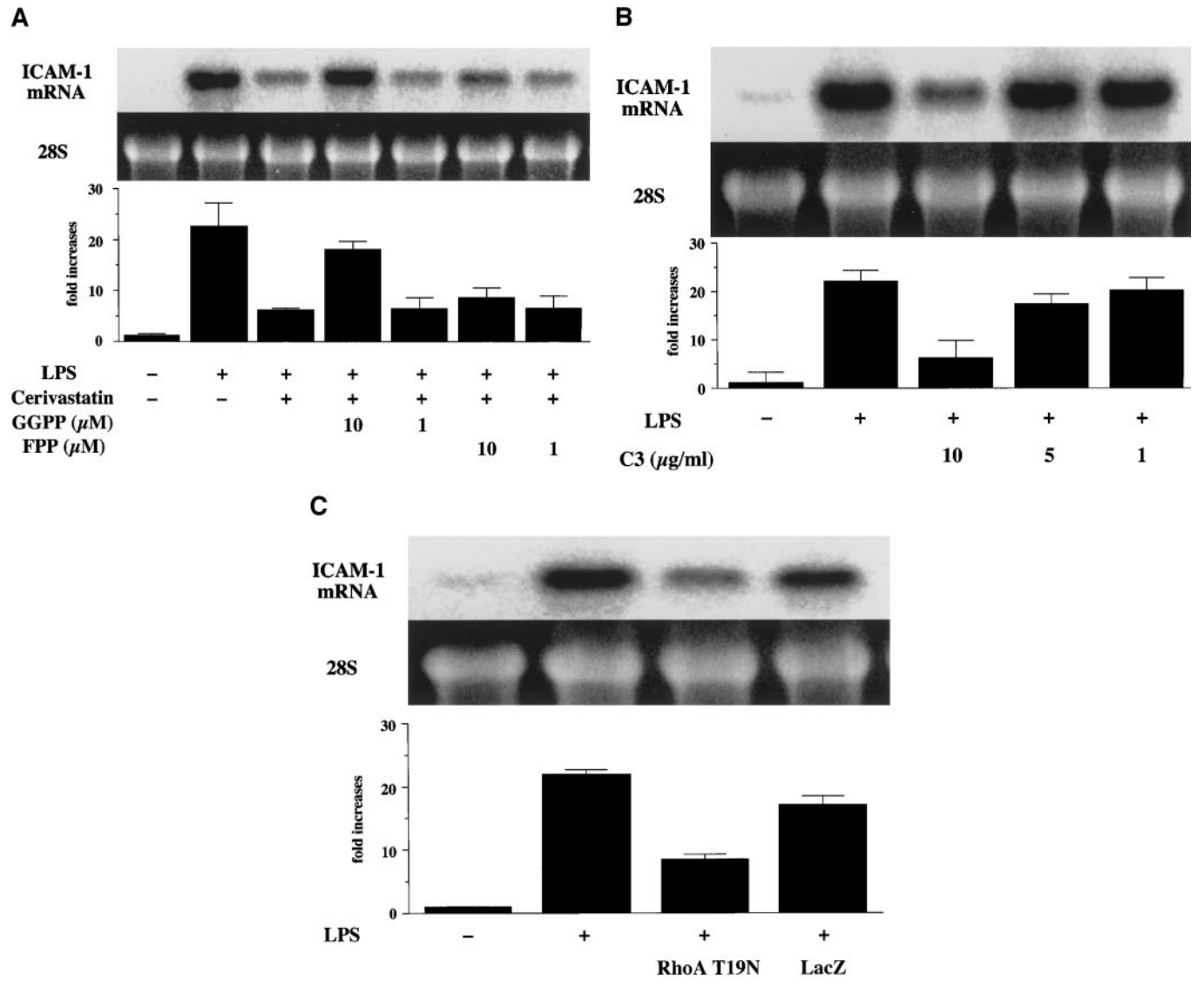

FIG. 3. Effects of Rho A modulators on ICAM-1 expression. (A) Confluent BAECs were pretreated with cerivastatin in the presence or absence of GGPP or FPP and subsequently stimulated with LPS stimulation for 10 h. (B) After pre-treatment with *Clostridium botulinum* C3 transferase (1–10 μg/ml) for 48 h, BAECs were stimulated with LPS for 10 h. (C) BAECs were infected with recombinant adenovirus expressing RhoA T19N or LacZ at 30 MOI for 48 h and subsequently stimulated by LPS for 10 h. Northern blot analysis was performed using cDNA probes of bovine ICAM-1. Representative results are shown in the upper panel. Fold increases of ICAM-1 mRNA expression are shown in the lower panel. The values are means ± SEM of at least 3 independent experiments.

Effects of Rho Modulators on ICAM-1 Expression

By addition of GGPP, Rho is geranylgeranylated and translocated to the membrane (20, 21). We investigated whether Rho is involved in the signal pathway of LPS-induced ICAM-1 expression. Prior to LPS stimulation, BAECs were treated with *Clostridium botulinum* C3 transferase (1–10 μg/ml), an exoenzyme which inactivates Rho by ADP-ribosylation, for 48 h. C3 decreased LPS-induced ICAM-1 mRNA expression in a dose dependent manner (Fig. 3B). Inhibition of Rho activity by overexpression of RhoA T19N, a dominant-negative mutant of RhoA, decreased LPS-induced ICAM-1 expression by 60% (Fig. 3C). In contrast, overexpression of LacZ showed a minimum effect on ICAM-1 expression. These findings indicate that RhoA associates with LPS-induced ICAM-1 expression pathway in BAEC, and that cerivastatin down-regulates this pathway by blocking geranylgeranylation of Rho.

DISCUSSION

The present results demonstrated that cerivastatin suppressed LPS-induced ICAM-1 mRNA expression in BAECs. HMG-CoA reductase inhibitors are shown to

possess modulatory effects on expressions of various genes including eNOS, metalloproteinase-9, and tissue factor (14, 22, 23). Recently, Laufs *et al.* revealed that HMG-CoA reductase inhibitors including mevastatin increase eNOS mRNA expression in endothelial cells. They demonstrated that HMG-CoA reductase inhibitors yield their effect on eNOS mRNA by increasing mRNA stability rather than acting on transcription (19). In accordance with their report, we found that eNOS mRNA was up-regulated by cerivastatin. NO is shown to inhibit LPS-induced ICAM-1 mRNA expression (18). However, as revealed by L-NAME treatment, the inhibitory effect of cerivastatin on LPS-induced ICAM-1 mRNA expression was not mediated by NO. Furthermore, the inhibitory effect was unlikely to be mediated by the effects of cerivastatin on ICAM-1 mRNA stability, since the inhibitory effect was specifically observed in the stimulation with LPS, but not with TNF-α (Fig. 1). Then we examined the possibility that cerivastatin exerts its inhibitory effect by directly acting on the signal pathway from LPS stimulation to ICAM-1 mRNA induction.

LPS, released from the outer membrane of Gram-negative bacteria, triggers cells to synthesize inflammatory mediators and induces expression or activation of adhesion molecules (4). Although the mechanisms by which LPS activates cells remain largely unexplored, both plasma LPS-binding protein (LBP) and CD14 have been shown to be required (24). Recent studies demonstrated that interaction between the ternary membrane CD14 (mCD14)-LPS-LBP complex and Toll-like receptor 2 (TLR2) on cell surface initiates the intracellular signal transduction for LPS in myeloid cells including monocytes (6). LPS induces adhesion of monocytes to endothelial cells, which is dependent on interaction between lymphocyte function-associated antigen-1 (LFA-1) on the surface of monocytes and its counter-receptor ICAM-1 on endothelial cells LPS is believed to activate both LFA-1 and ICAM-1. As for LFA-1 activation, a recent study demonstrated that Rho and phosphatidylinositol (PI) 3-kinase are located downstream of mCD14 (25). On the other hand, in endothelial cells, which do not express mCD14 on cell surface, the formation and subsequent cell surface binding of ternary soluble form CD14 (sCD14)-LPS-LBS complex are shown to induce intracelluar signal transduction including phosphorylation of MAP kinases (26, 27). However, the role of Rho in LPS-induced signal transduction in endothelial cells has not yet been examined.

HMG-CoA reductase inhibitors prevent the synthesis of important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). We examined the possibility that cerivastatin inhibits LPS-induced ICAM-1 mRNA expression by suppressing the synthesis of isoprenoid intermediates. As shown in Fig. 3A, the inhibitory effect of cerivastatin was reversed by co-treatment with GGPP. This finding indicates that the signal pathway of LPS-induced ICAM-1 expression is positively regulated by geranylgeranyl synthesis, and that cerivastatin suppresses this pathway by blocking geranylgeranyl synthesis.

The Rho family proteins, which include RhoA, RhoB, Rac, and Cdc42, are major substrates for post-translational modification by geranylgeranylation. Translocation to membrane by geranylgeranyl modification is necessary for activation of Rho. We found that inhibition of Rho by *Clostridium botulinum* C3 transferase or by overexpression of a dominant-negative RhoA decreased LPS-induced ICAM-1 expression. These results indicate that Rho is involved in LPS-induced signal transduction of ICAM-1 mRNA expression in BAEC, and that cerivastatin attenuates ICAM-1 mRNA expression by inhibiting Rho activation. The details of signal pathway in which Rho is involved are unclear, and further studies are necessary to clarify the mechanisms by which inhibition of Rho activation results in suppression of ICAM-1 expression. Recently, it was shown that Rho is required in endothelial cells for the assembly of stable adhesions with monocytes through regulation of clustering of E-selectin, ICAM-1, and VCAM-1, and these regulation is associated with actin cytoskeleton (28). Therefore, Rho is likely involved in LPS-induced leukocyte-endothelial adhesion by increasing both clustering and expression of ICAM-1.

The present results suggest that cerivastatin, an HMG CoA reductase inhibitor, may serve as a potential therapeutic tool toward inflammation in general, apart from atherosclerosis, by modulating expression of adhesion molecules.

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