Endothelium dysfunction, which is often defined as a decrease in NO bioavailability, is one of the earliest manifestations of endothelium-impaired function disorders, including atherosclerosis. Although improvement in NO bioavailability has been attributed to the lowering of serum cholesterol levels, recent studies suggest that HMG-CoA reductase inhibitors, statins, may have direct effects on NO bioavailability by little known mechanisms that are independent of serum cholesterol levels. The long-term effect of cerivastatin on NO release from endothelial cells was determined by using highly sensitive electrochemical microsensors and was correlated with endothelial NO synthase (eNOS) levels. To explore whether changes in isoprenoid synthesis affect NO bioavailability and eNOS expression, human endothelial cells were treated with cerivastatin, L-mevalonate (MVA; 1.5 mmol/L), geranylgeranylpyrophosphate (GGPP; 1 mg/mL) and farnesylpyrophosphate (FPP; 1 mg/mL). Cerivastatin increased spontaneous (by 53% ±6) and an eNOS-stimulated NO release (by 41 ±6% for calcium ionophore and by 47±5% acetylcholine) as well as eNOS expression (by 118 ±6%) in the same concentration-range. Cerivastatin-dependent increase in both NO release and eNOS expression was revealed after ~4 h of exposure reaching the maximum after ~10 h. Co-treatment with MVA or GGPP, but not FPP or LDL, reversed the effects of cerivastatin. These findings indicate that the long-term effect of cerivastatin resulting in enhanced NO bioavailability in endothelial cell is, at least in part, due to up-regulation of eNOS by blocking isoprenoids synthesis.

Key words: Nitric oxide, eNOS expression, endothelium, statins, cerivastatin
The HMG-CoA reductase inhibitors, or statins, are potent cholesterol lowering drugs. In addition to lowering cholesterol, recent clinical trials have demonstrated that statins significantly reduce cardiovascular mortality in patients with normal cholesterol concentrations (1-3). Mounting clinical and experimental evidence suggests that the protective effects of statins on vascular disease relate not only to improving lipid profiles but also to direct effects on endothelial function. Simvastatin has been reported to improve nitric oxide(NO)-dependent vasorelaxation in different vascular beds (4). Basal and stimulated endothelium-dependent forearm blood flow responses in hypercholesterolemic subjects are improved in 4 weeks of treatment with atorvastatin and simvastatin (5,6). Similarly, only 2 weeks of cerivastatin treatment improved endothelium-dependent vasodilation in man (7). These studies suggest a direct action of the statins on NO production in the endothelium. Indeed, we have recently shown using the direct method for quantification of biologically active (free) NO that cerivastatin, the most potent inhibitor of HMG-CoA reductase, facilitates NO release in endothelial cells rapidly (within the first few minutes) at concentrations as high as micromolar and in the long-term (after several hours) at more clinically relevant nanomolar concentrations (8,9).

There are a few possible cellular targets for statins which may lead to increasing endothelial NO synthase (eNOS) activity. Statins decrease cellular caveolin levels and attenuate the inhibition of eNOS by caveolin, resulting in increased NO production (10). Statins may regulate eNOS function through phosphorylation of the enzyme by a serine-threonine kinase (Akt) (11). Phosphorylation by Akt increases the affinity of eNOS and enhances the activity of eNOS. Statins also increase the recruitment of heat shock protein 90 (HSP90) to the eNOS complex (10). HSP90 is recruited to eNOS in response to different stimuli, and its binding is associated with rapid eNOS activation and NO release (12). In addition to affecting posttranslational regulatory mechanisms, statins increase eNOS transcription, stability, and protein level (13).

In fact, the inhibition of HMG-CoA reductase by statins leads to a decreased synthesis of cholesterol and also its precursors, which are isoprenoid products of mevalonate (MVA). These isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), provide lipophylic anchors which are essential for membrane attachment and biological activity of small GTP binding proteins from the Ras family (14). A decrease in isoprenylation of signaling molecules such as Rac, Ras and RhoA of the GTPase family leads to the modulation of various signaling pathways in endothelial cells which subsequently may change eNOS activity. For exerting their role in cell signal transduction, protein Ras and RhoA of the GTPase family must translocate from the cytoplasm to the cell membrane. This translocation requires FPP for Ras and GGPP for Rac and RhoA (15).
Because cerivastatin inhibits FPP and GGPP biosynthesis by inhibiting HMG-CoA reductase, we were prompted to analyze the effect of such inhibition on both NO release and expression of eNOS protein in endothelial cells. We also explored whether the long-term effect of cerivastatin on NO release from endothelial cells is caused by up-regulation of eNOS.

MATERIALS AND METHODS

Cell Culture

Human umbilical endothelial cells (HUVEC) were obtained from American Type Culture Collection (cat #CRL-1730) and cultured according to our previously described method (8). Cells were seeded in collagen-coated flasks and monitored until 75% of the cell clumps adhere (0.5-1 hour). Non-adhering cells were poured off and the adhering cells were incubated within minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) under 37°C at 5% CO₂ and 95% air. The medium was changed every two days. After 4-6 days, the primary cultures formed a confluent monolayer. Cell monolayer from stock flask was dissociated by exposure for 2-3 minutes at 24°C to 0.05% trypsin in 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate and 0.02% EDTA. When the cells rounded up, they were resuspended in MEM supplemented with 10% FBS and seeded at the final concentration of 2 x 10⁴ cells/35 mm dish. Four or 6 days later, when the cultures became confluent (4.5 x 10⁴ cells/35 mm dish) the medium was replaced with MEM containing 5% human lipoprotein-depleted serum (LPDS). The cells were further incubated at 37°C for 24 hours. In some experiments, cells were pretreated with different concentrations of cerivastatin for 1-24 hours with or without mevalonic acid (MVA), isoprenoids (GGPP and FPP), Low Density Lipoproteins (LDL) or selective endothelial and inducible NO synthase inhibitors.

Before the experiments, the reagents were prepared in the stock solutions as follows: calcium ionophore (10 mmol/L) was dissolved in DMSO, (LDL 10 mg/mL) was dissolved in 0.15 mol/L NaCl solution (pH=7.4), L-mevalonate (1.5 mmol/L) was dissolved in ethanol, GGPP and FPP (1 mg/mL each) were dissolved in 10 mmol/L aqueous NH₄OH solution (7:3). Ach, cerivastatin and the NO synthase inhibitors, N⁶-nitro-L-arginine methyl ester (L-NAME; eNOS selective inhibitor) and 1400W (iNOS selective inhibitor) were dissolved in MEM.

NO Measurement

A NO microsensor was prepared according to the procedures published previously (16). The sensor operated in a 3-electrode system, consisting of the sensor working electrode, a platinum wire (0.1 mm) counter electrode, and a standard calomel reference electrode. The current proportional to NO concentration was measured by a porphyrinic sensor, which operated in amperometric mode (EG&G PAR model 283 Potentiostat/Galvanostat was used) at constant potential of 0.75 V versus the standard calomel electrode. The response time used in these measurements was 0.1 ms, and the detection limit was 10⁻⁹ mol/L. The NO concentrations were determined from the measured current by means of a calibration curve (NO standard-saturated aqueous solution). The working electrode (NO sensor) was placed close to the surface (20±5 µm) of the cell membrane with the help of a computer-controlled micromanipulator.

At the end of the incubation period, 10 µl of test substance was injected to reach a final concentration in the cell medium as follows: 1 µmol/L calcium ionophore (CaI) A23187 or 1 µmol/L acetylcholine (Ach). The test substances were injected with a nanoinjector, which was
positioned at close proximity (5 to 7 µm) from the cell membrane. NO was measured as an increase of the current from its background level. The concentrations of the eNOS agonists, CaI and Ach, used in all experiments were selected on the basis of the dose-response curve (maximal response).

**Determination of Total eNOS Protein Expression**

HUVECs were washed twice with the homogenization buffer (20 mmol/L Tris-HCl, pH=7.5, 0.25 m/L sucrose, 10 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L dithiothreitol and protein inhibitors: 10 µg/mL pepstatin, 25 µg/mL aprotinin, 50 µg/mL leupeptin and 25 µg/mL trypsin), trypsinized, and centrifuged at 300 x g for 5 min at 4 ºC. After centrifugation, the cellular pellets were homogenized in the homogenization buffer containing 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate, followed by a 15-min incubation on ice and a 1-min sonication. Then, the samples were diluted with the ratio 1 : 2 in the Laemmli sample buffer (6.25 mmol/L Tris-HCl, pH=6.8, 25% glycerol, 2 % SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and boiled for 5 min at 95 ºC. Protein concentrations in the lysates were measured using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce Chemical Co.). Aliquots containing about 10 µg of protein were electrophoresed on 10% polyacrylamide gels at 100 V and subsequently transferred onto an activated piece of polyvinylidine difluoride membrane (immobilon-P; Millipore) at 90 mA overnight. After the proteins were transferred, the membrane was incubated with 0.1% glutaraldehyde for 10 min and washed three times with Tris-buffered saline (20 mmol/L Tris-HCl, pH=7.5, 0.5 mol/L NaCl) containing 0.1% Tween 20 (TBS-Tween buffer). The membrane was blocked with a buffer containing 5% milk powder, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20 for 1 h followed by three washes with TBS-Tween buffer. Blots were probed with anti-eNOS mouse monoclonal antibody in a dilution of 1 : 1000 in blocking buffer at room temperature for 2 h. After washing with TBS-Tween buffer, incubation with the secondary antibody (horseradish peroxidase-labeled anti-rabbit IgG, 1 : 1000) followed at room temperature for 1 h. Prestained markers (Bio-Rad Laboratories) were used for molecular mass determinations. eNOS protein was finally visualized using an enhanced chemiluminescence (ECL) detection system from Amersham. The density of ECL signals were quantitated with an Epson Perfection 2450 PHOTO image scanner using the public domain software package National Institute of Health.

**Fig. 1.** Dose-dependent effect of cerivastatin on peak NO release from endothelial cells after stimulation with CaI (solid bars) or Ach (dotted bars). Cells were exposed to various concentrations of cerivastatin for 24 hours and then treated with receptor-independent (CaI; 1 mmol/L) and receptor-dependent eNOS agonist (Ach; 1 mmol/L). n=6 for each bar; *P<0.01 vs control (without cerivastatin).
Image J 1.27 (available at http://rsb.info.nih.gov/nih-image/). In brief, after calibration of optical density, a density profile plot was generated for each band. Base lines and drop lines were drawn manually so that each peak defined a closed area. The area beneath the peaks was electronically calculated and expressed as pixel intensity per unit area. Determinations of optical density were performed on each band of at least three different blots.

**Data analysis**

Values are expressed as a mean ±SE, with a value of P<0.05 considered statistically significant. Statistical evaluation was performed by ANOVA followed by the Student unpaired t test. All analyses were made with the statistical software Microcal Origin (Microcal Software, Inc., Northampton, MA).

**RESULTS**

Treatment of endothelial cells with cerivastatin for 24 h increased NO release after stimulation with either Ach, a receptor-dependent NO synthase agonist, or CaI, a receptor-independent NO synthase agonist, in a dose-dependent manner (Fig. 1). The increase was similar for both agonists (by 41±4% for CaI and by 47±5% for Ach) reaching the maximal response at the concentration of cerivastatin above 0.1 nmol/L. As shown in Fig. 2, the incubation of endothelial cells with cerivastatin (1.0 nmol/L) resulted in the increase of either spontaneous or CaI-stimulated NO release (Ach data are not shown). More than 4 hours of incubation time was required to elicit a significant increase of NO release. After about 10 h of cell incubation with cerivastatin, NO that was released spontaneously as well as after the eNOS agonist stimulation, reached the maximum concentrations (11.2±0.3 nmol/L for spontaneous release and 740±25

![Fig. 2. Time-dependent effect of cerivastatin on spontaneous (dotted bars) and CaI(solid bars)-stimulated NO release from endothelial cells. Cells were exposed to 1.0 nmol/L of cerivastatin for 2-24 hours and then NO was measured without or after administration of Cal. *P<0.05 and **P<0.01 vs control (without cerivastatin).](image)
nmol/L for CaI stimulation). To determine whether the effect of cerivastatin on the eNOS agonists-stimulated NO release was associated with the changes in eNOS protein level, western blot assays using endothelial cells treated with cerivastatin (1.0 nmol/L) for 2, 4, 6, 10 and 24 h were performed (Fig. 3). Similar to the effect of cerivastatin on NO release, eNOS protein level increased significantly after 4 h of cell incubation with cerivastatin (1.0 nmol/L) and reached the maximal response after 10 h (118 ±6% above basal expression). The effect of cerivastatin on eNOS up-regulation in endothelial cells was concentration-dependent at the same concentration-range as for the cerivastatin

Fig. 3. Immunoblots showing time-dependent effect of cerivastatin (1.0 nmol) on eNOS protein level in endothelial cells. Representative western blot and bar graph show analysis of 4 separate experiments. *P<0.01 vs control (without cerivastatin).

Fig. 4. Immunoblots showing the effects of cerivastatin (0.01-10 nmol/L) alone or in combination with LDL-chol (1 mg/mL), MVA (10 nmol/L), GGPP (10 mmol/L) and FPP (10 mmol/L). Representative western blot and bar graph show analysis of 4 separate experiments. *P<0.05 and **P<0.01 vs control (without cerivastatin).
effect on an increase in NO release (Fig. 4). In contrast to eNOS, in all experiments there were no iNOS expressions detectable in endothelial cells. To assess whether cerivastatin-mediated up-regulation of eNOS protein level was due to inhibiting isoprenoid synthesis the endothelial cells were also co-treated for 24 h with MVA, GGPP, FPP and LDL-chol. Co-treatment with MVA and GGPP completely reversed the up-regulation of eNOS protein levels by cerivastatin. In contrast, co-treatment with FPP or LDL-chol did not significantly reverse the effects of cerivastatin on eNOS protein level. In the presence of isoprenoid derivatives MVA and GGPP, NO release was significantly decreased after stimulation with the eNOS agonist, 1 mmol/L CaI (for Ach data not shown) (Fig. 5). In the same experiments, FPP and LDL-chol did not affect NO release. Interestingly, treatment with the isoprenoid derivatives alone did alter neither basal eNOS protein level nor spontaneous and an eNOS-agonist stimulated NO release (data not shown). In order to confirm the involvement of the eNOS isoform in the effect of cerivastatin on NO release in endothelial cells, the changes of NO release stimulated with CaI were measured in the presence of 100 µmol/L of L-NAME. As expected, L-NAME inhibited CaI-stimulated NO release by about 75% either in the presence or absence of cerivastatin. In contrast, a selective iNOS inhibitor, (100 µmol/L 1400W), did not affect the eNOS agonist-stimulated NO release in the presence of cerivastatin.

**DISCUSSION**

This study demonstrates that cerivastatin enhances eNOS expression and NO release from endothelial cells. This effect was specific to the inhibition of
endothelial HMG-CoA reductase. The enhancement of NO production in endothelial cells corresponded to respective IC_{50} value of cerivastatin (17) and could be reversed with MVA. The increasing of both NO release and eNOS expression was reversed in the presence of GGPP, but not FPP, indicating that the process of geranylgeranylation in endothelial cells negatively regulates eNOS expression. In this regard, it has been previously reported that the Rho GTPase family, which includes RhoA, RhoB and Rac, are major substrates for post-translational modification by geranylgeranylation and that geranylgeranylation leads to the transfer of these Rho GTPases to the membrane (14,15). The membrane translocation of inactive, GDP-bound Rho causes activation of Rho through GDP/GTP exchange in the presence of guanine nucleotide exchange factor (18). Immunoblot analysis and GTP-radiolabeled binding assays revealed that statins can inhibit Rho membrane translocation and GTP binding activity, and both effects can be reversed in the presence of GGPP, but not FPP (13). Furthermore, it has been shown that inhibition of Rho by overexpression of a dominant-negative RhoA mutant, N19RhoA, causes an increase in eNOS expression. In contrast, direct activation of Rho by Escherichia coli cytotoxic necrotizing factor-1 leads to a decrease in eNOS expression (19). Thus, Rho may be considered as a negative regulator of eNOS expression. Also, of interest, treatment with MVA or GGPP alone did not affect both NO release and eNOS expression, suggesting maximal Rho geranylgeranylation under basal tissue culture conditions.

The data presented here show a good correlation between the degree of eNOS induction and the magnitude of the increase of NO release with respect to the dose- and time-exposure to cerivastatin. Our findings that improvement of NO release in endothelial cells by cerivastatin is mediated, at least partially, through up-regulation of eNOS expression is consistent with the previous reports showing that statins prevent the down-regulation of eNOS by oxidized LDL, TNF- and hypoxia (20,21). However, another potential mechanism by which statins may facilitate NO release from endothelial cells can be considered. We have recently demonstrated that statins themselves can efficiently scavenge superoxide at concentrations level required for stimulation of NO release (8,22). This dual, concurrent action of statins contributes to the prolongation of NO bioavailability in the cells and limits peroxynitrite formation and generation of the cytotoxic radicals. Inhibition of endothelial isoprenoid intermediates of the cholesterol synthesis may have many other important consequences on intracellular signaling pathways that influence eNOS activity beyond increasing expression of the enzyme. Inhibition of Rac1 geranylgeranylation and thereby Rac1-mediated NADPH oxidase activity by statins can attenuate angiotensin II-induced reactive oxygen species production (23,24). This antioxydative stress effect of statins may also modulate endothelial function by regulating caveolae formation. It has been demonstrated that reactive oxygen species dissociate eNOS and caveolin from caveolae-enriched plasma membranes (25). Another study showed that statins
rapidly activate Akt and eNOS phosphorylation on serine 1179 (11). A recent report by Eto et al. demonstrates that inhibition of Rho/Rho-kinase-dependent Akt dephosphorylation mediates the inhibitory effect of statin on endothelial tissue factor (26). In turn, farnesylation is necessary for targeting membrane proteins such as G-protein p21ras and thereby may act by receptor-mediated eNOS activity (27).

Endothelium dysfunction is a hallmark of the diseases comprehensively termed endothelium-impaired function disorders (e.g. atherosclerosis, essential hypertension, diabetes) and is characterized by reduced effective vascular NO action (28,29). One of the earliest recognizable benefits after treatment with HMG-CoA reductase inhibitors is the normalization of endothelium-dependent relaxation in atherosclerotic coronary arteries before significant lowering of serum cholesterol levels (6). Indeed, some other recent studies suggest that HMG-CoA statins may have direct effects on the vascular wall that are independent of serum cholesterol level (30). The present work shows the effect of cerivastatin on both NO release and eNOS expression in endothelial cells were not due to changes in extracellular levels of cholesterol, since all of the endothelial cells were exposed to a cholesterol-depleted culture medium. Furthermore, cholesterol supply in the form of LDL at a concentration sufficient to support cholesterol-dependent cellular processes (31) without decreasing uptake of L-arginine in endothelial cells (32) did not affect either NO release or eNOS expression. Thus, our results clearly indicate that the clinical applications of HMG-CoA reductase inhibitors may well extend beyond hypercholesterolemia and atherosclerosis but also to other endothelium-impaired function disorders associated with diminished eNOS activity.

In summary, we have shown that the improvement of the long-term NO production by cerivastatin in endothelial cells is due to inhibition of geranylgeranylation process (but not the blocking of synthesis of cholesterol itself) that finally leads to eNOS up-regulation. Because a decrease in NO production in endothelial cells may contribute to the pathogenesis of atherosclerosis and other endothelium-impaired function disorders, therapeutical approaches targeting on selective inhibition of synthesis of isoprenoid derivatives may prove to be beneficial. However, the mechanism of the rapid effect of statins on NO release (previously observed by our laboratory (8,22) to be within several minutes), which appears to be the typical eNOS agonist-like effect, needs to be further investigated.

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