Regulation of Endothelial Nitric Oxide Synthase and Endothelin-1 Expression by Fluvastatin in Human Vascular Endothelial Cells

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ABSTRACT—We investigated the effects of fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on endothelial vasoactive substances using human umbilical vein endothelial cells (HUVECs). Incubation of HUVECs with fluvastatin for 12 h increased endothelial nitric oxide synthase (eNOS) mRNA expression in a concentration-dependent manner (peak, 276 ± 38%, mean ± S.D., of the control, at 1.0 μM fluvastatin, P < 0.01). In addition, fluvastatin increased eNOS protein production (245 ± 51% of the control level, P < 0.05) as well as nitrite production (165 ± 35% of the control level, P < 0.01). In contrast, incubation of HUVECs with 1.0 μM fluvastatin for 12 h significantly reduced the production of endothelin-1 (ET-1) and preproET-1 mRNA expression in HUVECs (28 ± 1% and 39 ± 1% of the control level, respectively, P < 0.01). Our results suggest that fluvastatin might be involved in improvement of endothelial function and prevention of the progression of atherosclerosis.

Keywords: Fluvastatin, 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, Nitric oxide, Endothelin-1, Endothelial cell

The endothelium plays an important role in determining vascular tone through the production and release of a variety of vasodilator and vasoconstrictor substances acting on the underlying vascular smooth muscle (1). In atheromatous vessels, altered gene expression of endothelial vasoactive factors, such as endothelin-1 (ET-1) and nitric oxide (NO), have been suggested (2 – 4), resulting in impaired response of endothelium-mediated vasodilation (5). Indeed, ET-1 expression increases in the endothelium overlying atherosclerotic plaques, suggesting that ET-1 might be an important mediator of the atherogenic process (6). A diminished endothelium-derived NO has also been considered to play an important role in the atherogenic processes; monocyte adhesion to the endothelial surface, platelet aggregation, vascular smooth muscle cell proliferation and vasoconstriction (7 – 11). Thus, endothelial dysfunction is an important regulator of the atherogenic process as well as a marker of atherosclerosis.

Hypercholesterolemia is associated with impairment of endothelial function (12, 13), and treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors is believed to improve endothelial function as well as the survival of patients with coronary disease (14 – 17). Improvement of endothelial function by HMG-CoA reductase inhibitors is often explained by its serum cholesterol lowering effect. This is also supported by the fact that removal of low-density lipoprotein (LDL) by apheresis improves endothelium-dependent vasodilation in hypercholesterolemic patients (18). However, reduction of LDL does not appear to account entirely for the beneficial effects of HMG-CoA reductase inhibitors (19), and possible direct effects of these inhibitors have been suggested, which result in the restoration of endothelial function. Indeed, simvastatin prevents oxidized LDL-induced down-regulation of nitric oxide synthase (eNOS) mRNA expression and upregulation of preproET-1 mRNA expression in vascular endothelial cells (20, 21). In this study, we examined the modulatory effects of another HMG-CoA reductase inhibitor, fluvastatin, on the production of ET-1 and NO in cultured vascular endothelial cells.

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MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs; Sanko Junyaku, Tokyo) were seeded on plastic plates precoated with collagen (bovine type I; Sumitomo, Tokyo) and cultured in modified MCDB131 medium (Sigma, St. Louis, MO, USA) supplemented with 5% of fetal bovine serum (Gibco BRL, Long Island, NY, USA), 10 ng/ml of human basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA) and 10 units/ml of heparin (LEO Pharmaceutical Products, Ballerup, Denmark). All experiments were performed using confluent cells at second or third passage, and the medium was replaced with a fresh medium 24 h before the study. Then, we examined the effects of fluvastatin (Tanabe Seiyaku Co., Osaka) on eNOS and ET-1 expression using different concentrations (0.01, 0.1 and 1.0 μM, for 12 h) and different duration (1.0 μM for 6, 12 and 24 h). HUVECs incubated in 0.01% methanol (the final concentration of solvent for fluvastatin in the incubation medium) were used as vehicle controls. The trypan blue exclusion method revealed that fluvastatin did not affect the viability of HUVECs.

Isolation of RNA and cDNA cloning of eNOS and preproET-1

Total cellular RNA was isolated by the acid guanidinium-phenol-chloroform method using TRIZOL reagent (Gibco BRL). Human cDNAs for eNOS and preproET-1 were cloned by the reverse transcriptase-polymerase chain reaction (RT-PCR) as previously described (22). Total RNA isolated from HUVECs was reversely transcribed to cDNA using oligo dT primer. The following unique primers were synthesized for cloning of eNOS and prepro-ET-1 cDNA fragments: eNOS cDNA encoding 2651 to 2911 was subcloned in pGEM 11Z vector (Promega Co., Madison, WI, USA) of 116-bp length corresponding to 256 to 2911 of cDNA (American Type Tissue Culture Collection, Rockville, MD, USA) and cloned in pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). The ribonuclease (RNase) protection assay method was performed as described previously (22). Total cellular RNA and tRNA (Promega) (10 μg each) were hybridized at 45°C overnight with 32P-labeled eNOS and preproET-1 antisense cRNA probes (1 x 106 counts/min, each) combined with GAPDH antisense probe, synthesized by in vitro transcription using the linearized templates, and unhybridized probes were digested with RNase A (0.3 mg/ml, Sigma) and RNase T1 (30 units/ml, Gibco BRL) at 30°C for 1 h. RNases were then digested with proteinase K (0.5 mg/ml, Promega) at 37°C for 30 min. After phenol-chloroform extraction and ethanol precipitation, the hybridized probes protected against RNase digestion were denatured at 85°C for 3 min and electrophoresed on 6% polyacrylamide gels. Dried gels were exposed to X-ray films (Fuji photo, Tokyo) at −70°C to detect eNOS, preproET-1 and GAPDH mRNA expression. For quantitation of the expression of each mRNA, the autoradiographic bands were analyzed by computerized densitometry, using NIH Image (version 1.61; NIH Division of Computer Research and Technology, Bethesda, MD, USA). To standardize RNA content in each sample, values were expressed as percentages relative to the expression level of GAPDH mRNA.

Western blot analysis

Total protein was prepared with cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA and 1 mM sodium orthovanadate) containing 10 μg/ml leupeptin. Cell lysate (20 μg) was solubilized in Laemmli sample buffer (25) and then heated for 3 min at 100°C. Proteins from total cell lysate were separated by SDS-polyacrylamide (7.5%) gel electrophoresis and analyzed by immunoblotting. The blots were electrophoretically transfected to polyvinylidene difluoride (PVDF) (Immobilon-P; Millipore, Bedford, MA, USA) in blocking buffer (TBS) (20 mM Tris and 137 mM NaCl, pH 7.5). After extensive washing, these preparations were incubated with polyclonal anti-eNOS antibodies (1:1000 dilution; Transduction Laboratories, Lexington, KY, USA) in the blocking buffer at 4°C. The transferred polyvinylidene difluorides were then washed three times with TBS-T (20 mM Tris and 137 mM NaCl and 0.05% Triton-X 100, pH 7.5). Binding of antibodies was detected using horseradish-peroxidase-conjugated sheep anti-mouse IgG (Amersham, Little Chalfont, UK) and visualized with ECL chemiluminescence reaction reagents and ECL-hyperfilm (Amersham). eNOS bands were analyzed with NIH Image software and expressed relative to the control values.

Determination of nitrite concentration

When cells reached confluence, they were washed twice with phenol-red-free minimal essential medium (MEM,
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Gibco BRL) and incubated for 3, 6 and 12 h with 2 ml of a medium containing 2% of FBS in the presence or absence of fluvastatin (1.0 μM). At the end of incubation, supernatants of the media were collected and nitrite concentration was evaluated by the HPLC-Griess system (ENO-10; Eicom, Kyoto) as described previously (26). Previous studies have shown that nitrite, not nitrate, reflects NO production in a hemoglobin-free system (27). For this reason, we evaluated the concentration of nitrite as an index of NO formation in these experiments.

Quantitation of ET-1 levels
At the end of the incubation period, the supernatant was collected and ET-1 was measured by the double-antibody sandwich method with an enzyme-linked immunosorbent assay (ELISA) using an ET-1 ELISA kit (Wako Junyaku, Osaka).

Statistical analyses
Data are presented as the mean ± S.D. and analyzed by one-way ANOVA and the Dunnett post hoc test for data from the RNase protection assay or the Mann-Whitney U test for comparison between two groups. The unpaired t-test was also used when appropriate. A P value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Effect of fluvastatin on eNOS mRNA expression
Incubation of HUVECs with 0.01, 0.1 or 1.0 μM fluvastatin for 12 h resulted in increased expression of eNOS mRNA in a concentration-dependent manner. The maximum increase (276 ± 38% of the control level, P<0.01) was observed with 1.0 μM fluvastatin (Fig. 1). In a separate series of experiments designed to examine the serial changes in mRNA expression, we found that the maximum increase in eNOS mRNA expression (291 ± 25% of the control level, P<0.01) occurred at 6 h of incubation with 1.0 μM fluvastatin and no further increase was recognized at 24 h (262 ± 18% of the control level, P<0.05) of incubation (Fig. 2). No increase of eNOS mRNA was noted when 1.0 μM fluvastatin was used for 3 h.

Effect of fluvastatin on eNOS protein level
Incubation of HUVECs with 1.0 μM fluvastatin increased eNOS protein level at 12 h (Fig. 3A). Qualitative analysis indicated that eNOS protein level was significantly higher in the presence of fluvastatin (245 ± 51%, P<0.05) compared to the control (Fig. 3B).

Effect of fluvastatin on preproET-1 mRNA expression
Fluvastatin at concentrations of 0.01 and 0.1 μM had no effect on preproET-1 mRNA expression in HUVECs;
however, 1.0 μM of fluvastatin resulted in a significant reduction (39 ± 1.0% of the control level, \(P < 0.01\)) of preproET-1 mRNA expression (Fig. 4). The decrease in preproET-1 mRNA expression at 1.0 μM of fluvastatin was time-dependent, and the maximum decrease (28 ± 1.4% of the control level, \(P < 0.01\)) was noted after incubation for 24 h (Fig. 5).

**Fig. 2.** Time-dependent changes in eNOS mRNA expression in HUVECs induced by fluvastatin. Cells were incubated with 1.0 μM fluvastatin for the indicated periods and eNOS mRNA was assessed. The results were compared with the expression determined just before incubation (Cont.). A: The upper bands represent the expression of eNOS mRNA and the lower bands represent the expression of GAPDH mRNA. B: eNOS-to-GAPDH RNA ratios by densitometric analysis. Values are expressed as a percentage relative to the control and are presented as the mean ± S.D. of five experiments. **\(P < 0.01\), compared to the control.

**Fig. 3.** Effect of fluvastatin on immunoreactive eNOS content in HUVECs by Western blot analysis. Cells were incubated for 12 h in the presence (FLU) or absence (Cont.) of the agent. A: Western blot analysis. The level of eNOS was higher after incubation with fluvastatin (1.0 μM) than the control. B: Densitometric analysis. Values are expressed as a percentage relative to the control and are presented as the mean ± S.D. of three experiments. *\(P < 0.05\), compared to the control.

**Effect of fluvastatin on nitrite production**

As is shown in Fig. 6, nitrite production tended to increase along the incubation period in the vehicle control group. No significant difference in nitrite production was noted between the control and fluvastatin-treated group (1.0 μM) after 6 h of incubation. However, incubation of HUVECs with fluvastatin for 12 h resulted in a significant increase (165 ± 35% of the time-matched control group,
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**Fig. 4.** Effect of fluvastatin on preproET-1 mRNA expression in HUVECs. Cells were incubated with different concentrations of fluvastatin (FLU) for 12 h and preproET-1 mRNA was assessed by RNase protection assay. The results were compared with the expression determined in cells incubated with vehicle control medium (Cont.). A: The upper bands represent the expression of preproET-1 mRNA and the lower bands represent the expression of GAPDH mRNA. B: preproET-1-to-GAPDH RNA ratios by quantitative analysis with densitometry. Values are expressed as a percentage relative to the control and are presented as the mean ± S.D. of five experiments. **P<0.01, compared to the control.

**Fig. 5.** Time course changes in preproET-1 mRNA expression in HUVECs. Cells were incubated with 1.0 μM fluvastatin for the indicated periods followed by assessment of preproET-1 mRNA. The results were compared with the expression determined just before the incubation (Cont.). A: The upper bands represent the expression of preproET-1 mRNA and the lower bands represent the expression of GAPDH mRNA. B: preproET-1-to-GAPDH RNA ratios by densitometric analysis. Values are expressed as a percentage relative to the control and are presented as the mean ± S.D. of five experiments. **P<0.01, compared to the control.
Effect of fluvastatin on ET-1 production
Basal ET-1 production from HUVECs was 167 ± 30 pg/10⁶ cells during a 12-h period. Incubation of these cells with 1.0 μM of fluvastatin for 12 h resulted in a significant fall (28 ± 1.2% of the control level, *P<0.01*) in ET-1 production (Fig. 7).

**DISCUSSION**

Recent studies have shown that HMG-CoA reductase inhibitors exhibit a variety of ancillary effects; simvastatin inhibits platelet derived growth factor-induced DNA synthesis in human glomerular mesangial cells (28), fluvastatin inhibits proliferation of smooth muscle cells (29), and pravastatin inhibits monocyte chemotaxis and transendothelial migration (30). The major findings of the present study were that fluvastatin, an inhibitor of HMG-CoA reductase, 1) increased NO production that was accompanied by increased expression of both eNOS mRNA and protein, and 2) reduced ET-1 production with a corresponding decrease in the expression of preproET-1 mRNA in HUVECs. The effects of fluvastatin on NO and ET-1 production are likely to influence endothelial function, in addition to the main effect of reducing cholesterol levels.

The mechanisms by which fluvastatin causes opposite effects on eNOS mRNA and preproET-1 mRNA expression are unknown at present but may be related to inhibition of endothelial HMG-CoA reductase. Similar effects of other HMG-CoA reductase inhibitors, e.g., simvastatin, lovastatin and atorvastatin, on eNOS and preproET-1 expression have been demonstrated to be reversed in the presence of L-mevalonate (20, 21), and upregulation of eNOS expression by HMG-CoA reductase inhibitors has been reported to be the result of blockade of Rho geranylgeranylation by inhibition of L-mevalonate synthesis (31).

Our findings may also be explained by interaction between NO and ET-1 in cultured vascular endothelial cells. Recent studies have shown that ET-1 increases NO production by stimulating ET₂ receptors (32). However, endogenous NO is known to inhibit ET-1 production (33), being qualitatively in favor of our present observation that eNOS mRNA and nitrite accumulation increased, while preproET-1 mRNA and ET-1 decreased after fluvastatin.

The effects of fluvastatin on NO and ET-1 production observed in the present study could not be explained by its cholesterol lowering effect through inhibition of HMG-CoA reductase, because our experimental design included a cholesterol-free extracellular environment and no inhibition of hepatic HMG-CoA reductase. When similar reduction of serum LDL levels is achieved by HMG-CoA reductase inhibitors or by other modalities such as partial ileal loop bypass or treatment with cholestyramine, the clinical benefits are significantly higher in groups treated with HMG-CoA reductase inhibitors or by other modalities such as partial ileal loop bypass or treatment with cholestyramine, the clinical benefits are significantly higher in groups treated with HMG-CoA reductase inhibitors (34, 35). Furthermore, recent studies have shown that HMG-CoA reductase inhibitors prevent oxidized LDL-induced downregulation of eNOS mRNA expression and upregulation of preproET-1 mRNA expression (20, 21). O’Driscoll et al. (7) have demonstrated that improvement of endothelial function after 4 weeks of simvastatin treatment does not correlate with a decrease in serum cholesterol level. Therefore, fluvastatin, a HMG-CoA reductase inhibitor, might improve endothelial function not only through its serum cholesterol lowering effect but also by its direct effect on the endothelium.

Although endothelial dysfunction is a complex process, one of its main features is the impairment of endothelium-
dependent vasodilatation. Such impairment could bring about an imbalance between vasodilatation and vasoconstriction of blood vessels in vivo. Previous studies have indicated that disruption of this equilibrium is present in atherogenic vessels and is attributed to alterations in the L-arginine-NO-cGMP pathway (2, 3, 5). ET-1 might contribute to the modification of cellular responses during atherogenesis (4, 6, 36). In our experiments, fluvastatin increased NO production and reduced ET-1 production in HUVECs. These effects might be beneficial in endothelium-dependent vasodilatation and might act in preventing the progression of atherosclerosis.

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