Downregulation of Angiotensin II Type 1 Receptor by Hydrophobic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors in Vascular Smooth Muscle Cells

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Abstract—3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, so-called statins, reduce the relative risk of a major coronary event by lowering the serum cholesterol level. In addition, statins may confer beneficial effects by cholesterol-lowering independent mechanisms, which are incompletely characterized. Because angiotensin II (Ang II) plays crucial roles in the pathogenesis of cardiovascular diseases, we examined the effect of statins on the expression of the Ang II type 1 receptor (AT1-R) in cultured vascular smooth muscle cells (VSMCs). Cerivastatin and fluvastatin reduced the AT1-R mRNA and the AT1-R protein levels; however, pravastatin lacked this effect. Cerivastatin and fluvastatin suppressed the AT1-R promoter activity measured by luciferase assay but did not affect AT1-R mRNA stability, suggesting that the suppression occurs at the transcriptional level. Coincubation of VSMCs with mevalonate or geranylgeranyl pyrophosphate but not with farnesyl pyrophosphate reversed the cerivastatin-induced AT1-R downregulation. Overexpression of dominant-negative Rho A also suppressed AT1-R mRNA expression. Treatment with cerivastatin for 24 hours reduced the calcium response of VSMCs to Ang II. Taken together, statins downregulate AT1-R expression through a mevalonate-dependent, geranylgeranylation-dependent, and Rho A–dependent manner and attenuate the biological function of Ang II. Downregulation of AT1-R may contribute to the cholesterol-independent beneficial effect of statins on the cardiovascular system. (Arterioscler Thromb Vasc Biol. 2001;21:1896-1901.)

Key Words: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ■ angiotensin II receptors ■ vascular smooth muscle cells ■ mevalonate ■ geranylgeranylation

The most effective agents for lowering the plasma level of LDL cholesterol are 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, so-called statins. Many clinical trials have revealed that statins consistently reduce the relative risk of a major coronary event by ≈30%.1-4 It has also been demonstrated that statins are effective in the primary1 and secondary2-4 prevention of coronary heart disease, and the beneficial effects are extended to patients without high cholesterol levels.

Statins prevent oxidation of LDL,5 which plays a critical role in macrophage activation, and cholesterol lowering by statins significantly improves endothelial function.6 Recent results suggest that statins have additional effects independent of LDL lowering. Direct upregulation of endothelial NO synthase (eNOS) has been reported.7 Hydrophobic statins, such as cerivastatin and simvastatin, inhibit proliferation of vascular smooth muscle cells (VSMCs).8 These unique properties, along with a powerful LDL-lowering effect and excellent safety record, make these drugs quite useful in the treatment of hypercholesterolemia.

Angiotensin II (Ang II) plays crucial roles in the pathogenesis of atherosclerosis and hypertension.9 Ang II causes hypertrophy, the production of extracellular matrix, and the expression of various growth factors in VSMCs.10 Although 2 Ang II receptor isoforms designated type 1 receptor (AT1-R)11 and type 2 receptor (AT2-R)12 have been cloned, most of the cardiovascular effects are mediated by the AT1-R. AT1-Rs of VSMCs are increased in atherosclerotic lesions and the neointima after balloon injury.13 ACE inhibitors and AT1-R antagonists suppress neointimal formation.14 These results suggest that upregulation of AT1-R and enhancement of Ang II actions in the vessel wall contribute to athogenesis.

The aim of the present study was to determine whether statins affect the AT1-R gene expression in VSMCs. We demonstrate that cerivastatin and fluvastatin, but not prava-
statin, negatively regulate AT1-R gene expression. Cerivastatin and fluvastatin also reduced calcium response to Ang II. Although the mechanism of the differential effect of statins on AT1-R expression has not been determined, suppression of AT1-R expression may be one of the cholesterol-independent effects that may contribute to the clinical benefit of statins.

Methods

Reagents
Cerivastatin, pravastatin, and fluvastatin were provided by Bayer Co, Sankyo Pharmaceutical Co, and Novartis Pharmaceutical Co, respectively. These statins were dissolved in distilled water. BSA, ionomycin, geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), and mevalonate were purchased from Sigma Chemical Co. DMEM and FBS were purchased from Gibco-BRL. Botulinum C3 exotoxin was purchased from Calbiochem. Y27632 was a generous gift from Welfide Pharmaceutical Co. [α-32P]dCTP and [3H]Sar1,Ile8-Ang II were purchased from DuPont New England Nuclear. Fura 2-AM (an acetoxyethyl ester form of fura 2) was purchased from Dojindo. Other chemical reagents were purchased from Wako Pure Chemicals, unless mentioned specifically. Dominant-negative and constitutively active forms of Rho A were provided by Dr Kozo Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan). Dominant-negative Rho A was cloned and purchased from Dojido. Other chemical reagents were purchased from Sigma Chemical Co. DMEM and FBS were purchased from GIBCO-BRL. Botulinum C3 exotoxin was purchased from Calbiochem. Y27632 was a generous gift from Welfide Pharmaceutical Co. [3H]Sar1,Ile8-Ang II was purchased from DuPont New England Nuclear. Fura 2-AM (an acetoxyethyl ester form of fura 2) was purchased from Dojindo. Other chemical reagents were purchased from Wako Pure Chemicals, unless mentioned specifically.

Cell Culture
VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and maintained as described previously.15 VSMCs were grown to confluence in DMEM with 10% FBS, growth-arrested in DMEM with 0.1% BSA for 2 days, and then used for the experiments. Passages between 6 and 12 were used.

Northern Blot Analysis
Total RNA was prepared according to an acid guanidinium thiocyanate–phenol–chloroform extraction method,16 and Northern blot analysis of AT1-R and 18S rRNA was performed as described previously.17 The radioactivity of hybridized bands of AT1-R mRNA and 18S rRNA was quantified with a MacBAS Bioimage Analyzer. The ratio of AT1-R mRNA level to RNA expression is shown in the table (n = 6). The ratio of unstimulated cells was designated 100%. Con indicates control.

Measurement of Cell Viability
Confluent VSMCs were serum-deprived for 48 hours and then treated with cerivastatin, pravastatin, and fluvastatin. After 24 hours of incubation, these cells were harvested with trypsin-EDTA and stained with 0.4% trypan blue. The total and dead cells were counted with a hemocytometer.

Estimation of the Number of AT1-R Binding Sites
Confluent VSMCs in 24-well dishes were cultured in DMEM supplemented with 0.1% BSA for 48 hours and incubated with or without 10 μmol/L cerivastatin or fluvastatin for 24 hours. The number of AT1-R binding sites was estimated through the method (Transfast, Promega Co) according to the manufacturer’s instructions.

Measurement of AT1-R Gene Promoter Activity
The AT1-R promoter–luciferase fusion DNA construct (−980 bp) has been described previously.18 VSMCs (5 × 10^4) were prepared in a 6-cm tissue culture dish. After 48 hours, 5 μg of the AT1-R promoter–luciferase fusion DNA construct and 2 μg of the LacZ gene driven by the simian virus 40 promoter-enhancer sequence were introduced to VSMCs via the DEAE dextran method according to the manufacturer’s instructions (Promega). These cells were cultured in DMEM supplemented with 10% FBS for 24 hours and stimulated with cerivastatin, pravastatin, or fluvastatin in DMEM containing 0.1% BSA for 24 hours. The luciferase activity was measured and normalized by β-galactosidase activity as described previously.19

Infection of Adenovirus
VSMCs grown to confluence were washed with PBS 3 times. Then the cells were incubated with AdDN Rho A under gentle agitation for 2 hours at room temperature. After infection, the cells were washed 3 times, cultured in DMEM with 0.1% BSA for 2 days, and then used for the experiments. Multiplicity of infection indicates the amount of virus per cell added to the culture dish.

Measurement of Intracellular Calcium Response
VSMCs were preincubated in DMEM containing 5 μmol/L fura 2-AM for 1 hour and then incubated with or without cerivastatin or fluvastatin for 10 minutes (short-term treatment). Alternatively, VSMCs were preincubated with cerivastatin or fluvastatin for 24 hours before fura 2-AM loading (long-term treatment). Then VSMCs were stained with 0.4% trypan blue. The total and dead cells were counted with a hemocytometer.

Figure 1. Effect of statins of AT1-R mRNA expression and promoter activity. A, VSMCs were incubated with 10 μmol/L cerivastatin (C), pravastatin (P), or fluvastatin (F) for 24 hours. Total RNA was isolated, and the expression of AT1-R mRNA and 18S rRNA was determined by Northern blot analysis. A representative autoradiograph is shown. The bands of AT1-R mRNA and 18S rRNA were quantified with a MacBAS Bioimage Analyzer. The ratio of AT1-R mRNA level to RNA expression is shown in the bar graph (n = 6). The ratio of unstimulated cells was designated 100%. Con indicates control. B, The AT1-R promoter–luciferase fusion DNA was introduced to VSMCs, and the cells were stimulated with 1 or 10 μmol/L cerivastatin (C1 and C10, respectively), pravastatin (P1 and P10, respectively), or fluvastatin (F1 and F10, respectively) for 24 hours. Then luciferase and β-galactosidase activities were measured. The relative luciferase activity of unstimulated VSMCs (control) was set as 100%. Values (mean ± SEM) are expressed as a percentage of control culture (100%, n = 6). *P < 0.01 vs control. C, VSMCs were incubated with 10 μmol/L cerivastatin for the varying time periods as indicated. Expression of AT1-R mRNA was examined by Northern blot analysis. The bar graph shows the ratio of AT1-R mRNA to 18S rRNA (n = 6). D, VSMCs were incubated with cerivastatin at varying concentrations, as indicated, for 24 hours. Expression of AT1-R mRNA was examined by Northern blot analysis (n = 6).

Measurement of AT1-R Gene Promoter Activity
The AT1-R promoter–luciferase fusion DNA construct (−980 bp) has been described previously.18 VSMCs (5 × 10^4) were prepared in a 6-cm tissue culture dish. After 48 hours, 5 μg of the AT1-R promoter–luciferase fusion DNA construct and 2 μg of the LacZ gene driven by the simian virus 40 promoter-enhancer sequence were introduced to VSMCs via the DEAE dextran method according to the manufacturer’s instructions (Promega). These cells were cultured in DMEM supplemented with 10% FBS for 24 hours and stimulated with cerivastatin, pravastatin, or fluvastatin in DMEM containing 0.1% BSA for 24 hours. The luciferase activity was measured and normalized by β-galactosidase activity as described previously.18
were washed with buffer containing 5 mmol/L KCl, 10 mmol/L HEPES, 5.5 mmol/L glucose, 1 mmol/L MgCl₂, 135 mmol/L NaCl, and 1 mmol/L CaCl₂, and stimulated with 100 nmol/L Ang II. Intracellular calcium concentration ([Ca²⁺]) was measured with a fluorescence spectrophotometer (CAM-230, Japan Spectroscopic) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. The fluorescence data were expressed as percentages of the values at rest and at the peak response obtained with 25 μmol/L ionomycin, assigned to be 0% and 100%, respectively.

Statistical Analysis

Statistical analyses of the relative AT₁-R mRNA expression were performed with 1-way ANOVA and the Fisher test if appropriate. The difference of the dissociation constant (K₈) and the AT₁-R binding site (B_max) was compared by the Mann-Whitney U test. Degradation of AT₁-R mRNA was analyzed by 2-way ANOVA. Data are shown as mean ± SEM. A value of P<0.05 was considered to be statistically significant.

Results

Suppression of AT₁-R mRNA Expression by Cerivastatin and Fluvastatin

VSMCs were incubated with 10 μmol/L of cerivastatin, pravastatin, or fluvastatin for 24 hours. Figure IA shows that the expression level of AT₁-R mRNA in VSMCs incubated with cerivastatin or fluvastatin was significantly reduced compared with that of unstimulated (control) cells (P<0.01). However, pravastatin lacked this effect. Even 1 mmol/L of pravastatin did not affect AT₁-R mRNA expression (data not shown). Figure IB shows that incubation with 10 μmol/L of cerivastatin or fluvastatin reduced the promoter activity of the AT₁-R gene measured by luciferase assay (P<0.05). Consistent with the result of Northern blot analysis, pravastatin did not affect AT₁-R promoter activity. Figure IC shows the time course of the AT₁-R mRNA expression incubated with 10 μmol/L of cerivastatin. Suppression of AT₁-R mRNA was observed after 6 hours of incubation. However, pravastatin did not affect AT₁-R mRNA expression during 24 hours of incubation (data not shown). Cerivastatin dose-dependently suppressed AT₁-R mRNA expression (Figure ID). Fluvastatin also reduced the AT₁-R mRNA expression with a time course similar to that of cerivastatin (data not shown).

Cerivastatin Reduced AT₁-R Binding Sites

After 24 hours of incubation with cerivastatin or fluvastatin, AT₁-R binding sites were determined by radiolabeled ligand binding assay. Figure 2 shows a saturation curve and Scatchard plot analysis of the binding of [¹²⁵I]Sar¹,Ile⁸-Ang II to unstimulated (control) and cerivastatin- or fluvastatin-treated cells. Binding to control cells revealed a B_max value of 0.77 pmol/mg protein and a K_d value of 4.5 nmol/L. On the other hand, cerivastatin- and fluvastatin-treated cells showed significantly reduced B_max (0.33 and 0.44 pmol/mg protein, respectively; P<0.05) and statistically unchanged K_d (4.2 and 4.4 nmol/L, respectively). These data indicate that cerivastatin significantly reduced the AT₁-R number without changing the affinity of AT₁-R to Ang II.
Because statins were reported to have a proapoptotic effect in VSMCs, we measured the viability of VSMCs by trypan blue exclusion assay. Treatments of VSMCs with 10 μmol/L of cerivastatin, pravastatin, or fluvastatin for 24 hours did not show statistically significant differences in cell viability compared with control (percentage of viable cells: control 97.2±1.5%, cerivastatin 96.3±0.7%, pravastatin 93.0±0.7%, and fluvastatin 95.8±1.0%; n=6).

**Effect of Cerivastatin on AT₁-R mRNA Stability**

We examined whether statins affected the AT₁-R mRNA stability. VSMCs were incubated with or without cerivastatin or fluvastatin (10 μmol/L) for 12 hours, and then actinomycin D (5 μg/mL) was added. Figure 3A shows that the degradation rate of AT₁-R mRNA is not significantly different between control and cerivastatin- or fluvastatin-treated cells. These data indicate that statins do not change AT₁-R mRNA stability.

To examine whether cerivastatin-induced downregulation of AT₁-R mRNA requires de novo protein synthesis, we examined the effect of cycloheximide (CHX, 10 μg/mL; Figure 3B). Incubation with CHX alone for 12 hours upregulated AT₁-R mRNA expression. Cerivastatin failed to suppress the AT₁-R mRNA expression in the presence of CHX. These data suggest that cerivastatin-induced AT₁-R downregulation was dependent on de novo protein synthesis.

**Effect of Mevalonate on Cerivastatin-Induced AT₁-R Downregulation**

To confirm that the effect of cerivastatin on AT₁-R expression was mediated by the inhibition of HMG CoA reductase, VSMCs were incubated with cerivastatin in the presence of mevalonate. Cerivastatin failed to downregulate AT₁-R mRNA expression in the presence of mevalonate, suggesting that downregulation of AT₁-R mRNA expression is due to inhibition of the cholesterol biosynthetic pathway downstream from mevalonate (Figure 4A).

Next, we examined the effects of GGPP and FPP, which are important isoprenoids derived from mevalonate. As shown in Figure 4B, GGPP but not FPP was able to prevent cerivastatin-induced AT₁-R downregulation. FPP at 50 μmol/L did not affect cerivastatin-induced AT₁-R downregulation (data not shown).

It is reported that inhibition of geranylgeranylation by statin inhibited the activity of the Rho small G protein. Therefore, we examined the effect of overexpression of the dominant-negative form of Rho A on AT₁-R mRNA expression. AdDN Rho A suppressed AT₁-R mRNA expression in a manner comparable to that of cerivastatin (Figure 4C). We also used C3 exotoxin (10 μg/mL), a specific inhibitor of Rho. C3 exotoxin significantly suppressed AT₁-R mRNA expression (data not shown), and overexpression of constitutively active Rho A reversed the cerivastatin-induced AT₁-R mRNA expression (Figure 4D), suggesting that cerivastatin decreased AT₁-R mRNA expression through inhibition of Rho A.

**Cerivastatin Decreased Calcium Response to Ang II**

Finally, we examined whether statin-induced AT₁-R downregulation decreased the response of VSMCs to Ang II stimulation. VSMCs were preincubated with cerivastatin or fluvastatin for 10 minutes or 24 hours. The VSMCs were stimulated with 100 nmol/L Ang II, and [Ca²⁺]i was measured. A brief preincubation (10 minutes) with these statins did not affect Ang II–induced calcium response (Figure 5A). Ang II–induced maximal [Ca²⁺]i increases were 38.1±1.5%, 39.0±1.4%, and 38.0±0.93% (in percentage of maximum fluorescence induced by ionomycin treatment) in control, cerivastatin-treated, and fluvastatin-treated VSMCs. However, long-term incubation (24 hours) with statins significantly decreased the calcium response to Ang II (Figure 5B). The Ang II–induced maximal [Ca²⁺]i increase in control VSMCs was 39.8±0.94%; however, increases in cerivastatin- and fluvastatin-treated cells were 28.3±1.9% (P<0.01 versus control) and 30.0±1.3% (P<0.01 versus control), respectively.

**Discussion**

In the present study, we demonstrated that cerivastatin and fluvastatin reduced the expression of AT₁-R in cultured VSMCs. Cerivastatin and fluvastatin reduced AT₁-R pro-
FIGURE 5. Effect of statins on intracellular calcium response to Ang II. A, VSMCs were pretreated with or without cerivastatin or fluvastatin (10 μmol/L) for 10 minutes. B, VSMCs were pretreated with or without cerivastatin or fluvastatin (10 μmol/L) for 24 hours. Then these VSMCs were stimulated with 100 nmol/L Ang II, and [Ca2+]i was measured. A representative record is shown. Ten minutes of treatment with statins did not affect Ang II–induced calcium response. However, long-term treatment with statins significantly (P<0.01) reduced the calcium response to Ang II compared with the control (n=4).

motor activity without affecting AT1-R mRNA stability, suggesting that these statins suppress AT1-R gene expression at the transcriptional level rather than at the posttranscriptional level. In addition, the Ang II–induced calcium response was attenuated by incubation with cerivastatin. It is unlikely that statins directly inhibited the calcium current induced by Ang II, because the Ang II–induced calcium response was not affected by a brief incubation with cerivastatin or fluvastatin. Therefore, the decreased response of [Ca2+]i to Ang II after a 24-hour incubation of statins probably reflects the reduction of AT1-R numbers.

The failure of pravastatin to downregulate AT1-R expression may be due to the hydrophilicity of the drug, which prevents penetration of this drug through the plasma membrane. Therefore, pravastatin, for which the presence of a specific carrier is suggested in hepatocytes,21 may fail to inhibit cholesterol synthesis in cultured VSMCs. Although statins are reported to inhibit VSMC proliferation,8 pravastatin lacked this effect, and the same mechanism is proposed. IC50 of pravastatin for the inhibition of cholesterol synthesis is 100 to 1000 times higher in VSMCs than hepatocytes.8 However, cholesterol-independent improvement of endothelial function is reported in primates receiving a dose of pravastatin that does not change the LDL level.22 Thus, the hydrophobicity of statin is not a sole determinant of pleiotropic effect.

Although induction of apoptosis by statins is reported,19 the viability of VSMCs was not affected by treatment with statins in our experimental condition. These data suggest that cell death does not account for the downregulation of AT1-R expression by statins.

The concentration of the statin that we used in this in vitro study is higher than that achieved in the plasma by oral administration in humans. Therefore, it is not clear at this point whether downregulation of AT1-R contributes to the cardioprotective effects of statins observed in the clinical trials. Recently, a report showed that cerivastatin ameliorated renal injury in rats double transgenic for human renin and angiotensinogen, in which increased Ang II caused cardiac hypertrophy and renal injury.23 That report also showed that the anti-inflammatory and antiproliferative effects of cerivastatin were independent of blood pressure or cholesterol lowering. These data suggest that statin may inhibit the renin-angiotensin system in vivo, which may contribute to the beneficial effects of statin independent of the cholesterol level.

In addition to the cholesterol-lowering effect, statins have a pleiotropic effect, such as the inhibition of tissue factor expression,24 scavenger receptor expression,25 and superoxide generation.26 Mevalonate, of which HMG CoA reductase inhibitor inhibits the production, is the precursor not only of cholesterol but also of many nonsteroidal isoprenoid compounds. Isoprenoids are necessary for cell division and proliferation and are assumed to be responsible for these pleiotropic effects that are independent of the cholesterol-lowering effect of statins.27 The addition of mevalonate or GGPP reversed statin-induced AT1-R downregulation. Therefore, the HMG CoA–mevalonate–GGPP pathway is responsible for AT1-R downregulation. One of the geranylgeranylated proteins that may be important is the Rho small GTP-binding protein. As expected, overexpression of dominant-negative Rho A reduced AT1-R mRNA, suggesting that basal Rho A activity plays an important role in AT1-R gene expression. We incubated VSMCs for 48 hours with AdDN Rho A, because it is generally believed that 48 hours of incubation is necessary to achieve satisfactory expression of the transduced gene by adenovirus.

Recently, potential effector proteins for Rho, such as Rho kinase, citron kinase, and rhophilin, have been identified.28 We have not identified the downstream target of Rho protein that mediates AT1-R downregulation. Y27632, an inhibitor for Rho kinase, did not decrease AT1-R mRNA (data not shown), suggesting that Rho kinase may not be responsible for cerivastatin-induced AT1-R downregulation. Further study is necessary to identify the target molecule of Rho that is responsible for AT1-R downregulation.

The induction of eNOS gene expression by simvastatin involves Rho-mediated stabilization of eNOS mRNA.27 However, cerivastatin or fluvastatin did not affect AT1-R mRNA stability, and promoter activity of AT1-R gene was suppressed by these statins. These data suggest that cerivastatin and fluvastatin may suppress the transcription of the AT1-R gene. Cerivastatin-induced AT1-R downregulation was inhibited by CHX, suggesting that this process requires de novo protein synthesis. At present, the nature of the protein that is induced by statin has not been determined.

In conclusion, we showed in the present study that cerivastatin and fluvastatin reduced AT1-R expression in VSMCs. The downregulation of AT1-R expression depends on the mevalonate–GGPP–Rho A pathway. Downregulation of AT1-R, at least in part, may contribute to the clinical benefit of the HMG CoA reductase inhibitor in the treatment of coronary artery disease.

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References


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