Fluvastatin Enhances the Inhibitory Effects of a Selective Angiotensin II Type 1 Receptor Blocker, Valsartan, on Vascular Neointimal Formation

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Background—The present studies were undertaken to investigate the potential effect of a hydroxymethylglutaryl coenzyme A reductase inhibitor (statin) to enhance the inhibitory effect of an angiotensin (Ang) II type 1 (AT1) receptor blocker (ARB) on vascular neointimal formation and to explore the cellular mechanism of cross-talk of the AT1 receptor and statin in vascular smooth muscle cells (VSMCs).

Methods and Results—Neointimal formation and the proliferation of VSMCs induced by cuff placement around the femoral artery were significantly inhibited by treatment with an ARB, valsartan, at a dose of 0.1 mg · kg⁻¹ · d⁻¹ and with fluvastatin at a dose of 1 mg · kg⁻¹ · d⁻¹, which did not influence mean arterial blood pressure or plasma cholesterol level, whereas valsartan or fluvastatin alone at these doses did not affect neointimal formation or the proliferation of VSMCs. Pretreatment with fluvastatin (~5 μmol/L) for 24 hours significantly inhibited Ang II (1 μmol/L)–mediated DNA synthesis and c-fos promoter activity in cultured VSMCs. Moreover, pretreatment of VSMCs with fluvastatin significantly inhibited Ang II–mediated extracellular signal-regulated kinase (ERK) activation and tyrosine- and serine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3. AT1 receptor–mediated recruitment of Rac-1 to Janus kinase (Jak) family/STATs was also inhibited by fluvastatin. Consistent with these in vitro results, phosphorylation of ERK, STAT1, and STAT3 was attenuated by the coadministration of valsartan and fluvastatin even at low doses in vivo.

Conclusion—These results suggest that the cholesterol-independent inhibition of AT1 receptor–mediated VSMC proliferation by statins may contribute to the beneficial effects of statins combined with an ARB on vascular diseases. (Circulation. 2003;107:106-112.)

Key Words: angiotensin ■ hydroxymethylglutaryl coenzyme A reductase inhibitors ■ remodeling

The major cardiovascular actions of angiotensin (Ang) II have been reported to be mediated by the Ang II type 1 (AT1) receptor.³ It is conceivable that the renin-angiotensin system (RAS) plays a major role in neointimal thickening, which is well characterized in animal models. Arterial neointimal thickening is an important process in the development of atherosclerosis, bypass graft failure, and restenosis after angioplasty. In injured arteries, components of RAS are upregulated. We have developed a mouse model of vascular disease induced by polyethylene cuff placement around the femoral artery, in which the AT1 receptor as well as angiotensin-converting enzyme (ACE) is upregulated, followed by neointimal thickening.² Moreover, we have reported that a selective AT1 receptor blocker (ARB), valsartan, attenuated neointimal formation and vascular smooth muscle cell (VSMC) proliferation, with a decrease in inflammation.³

Recent large clinical trials have demonstrated that the therapeutic use of 3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors (statins) results in a decreased incidence of ischemic stroke and myocardial infarction and a reduction of mortality in hypercholesterolemic subjects. The effects of statins have been mainly attributed to their cholesterol-lowering properties, but there is growing evidence that some beneficial effects of these agents may be independent of plasma cholesterol level.⁴,⁵ Recent studies have demonstrated that statins may prevent Ang II–induced cellular and organ damage, such as the production of reactive oxygen species in VSMCs, cardiac hypertrophy, and end-organ damage.⁶–⁸ Therefore, we speculated that statins may enhance the effect of an ARB to improve vascular remodeling. The present study was undertaken to test this idea in a mouse vascular injury model and to examine the possibility that statins may inhibit VSMC proliferation by antagonizing AT1 receptor–mediated signaling.

Methods

Animals, Treatment, and Morphometric Analysis
Adult male C57BL/6J mice (10 to 12 weeks of age; 25 to 30 g; Clea Japan) were used in the present study. The Animal Studies Commit-
tee of Ehime University approved the following experimental protocol. The surgical procedure for the cuff-induced vascular injury model in the femoral artery, morphometric analysis, and measurement of DNA synthesis were performed according to the methods described previously.\(^2\)\(^3\) Briefly, a polyethylene tube (2-mm-long PE 90, Becton Dickinson) was cut longitudinally to open the tube, loosely placed around the left femoral artery, and closed with sutures. The middle segment of the artery was cut into 3 subserial cross-sections with 5-µm thickness at an interval of 0.3 mm. DNA synthesis in the injured artery was assayed by the incorporation of bromodeoxyuridine (BrdU). The sections were counterstained with hematoxylin, and the BrdU index (the ratio of BrdU-positive nuclei/total nuclei) was calculated. The mean value of 3 sections was taken as the value for each animal. Valsartan (provided by Novartis Pharma AG, Basel, Switzerland), an AT\(_1\) receptor-selective ARB, was administered using an osmotic minipump (model 1002, Alza Corporation) that was implanted intraperitoneally at the same time as cuff placement, as previously described.\(^3\) Fluvastatin (provided by Novartis Pharma AG, Basel, Switzerland) was dissolved in water and administered daily by gavage at 0.1 mL. Plasma cholesterol level was measured by the cholesterol oxidase method (Cholesterol E-test, WAKO Chemical Industries).

Cell Culture and Measurement of DNA Synthesis by \[^{3}H\]Thymidine Incorporation

Adult rat aortic VSMCs were prepared as previously described.\(^9\)\(^10\) Cells at passage 3 to 8 were used for the experiments. DNA synthesis was assayed by measuring \[^{3}H\]thymidine incorporation as previously described.\(^9\) AT\(_1\) or Ang II type 2 (AT\(_2\)) receptor binding was measured as previously described.\(^1\)\(^0\) Briefly, the cells were incubated for 1 hour at 37°C with 0.1 nmol/L \[^{125}\text{I}\]Sar\(_1\), Ile\(_8\)Ang II (Du Pont NEN Research Product) in the absence or presence of 1 µmol/L valsartan or 1 µmol/L PD123319 (Research Biochemical International). AT\(_1\) receptor binding was calculated as the difference between the total count and the count from samples incubated with valsartan. AT\(_1\) receptor binding was determined by subtracting the count of samples incubated with PD123319 from the total count.

Transfection and c-fos Promoter Assay

VSMCs were seeded in 6-well plates and transfected with the c-fos-luciferase reporter gene (p2FTL, 1 µg) using LipofectAMINE PLUS reagent (GIBCO-BRL) according to the manufacturer’s instructions. This gene consists of 2 copies of the c-fos S\(_{5}\)-regulated enhancer element (−357 to −276), the herpes simplex virus thymidine kinase gene promoter (−200 to 70), and the luciferase gene.\(^1\)\(^1\) To ensure the efficiency of equivalent transfection, we cotransfected the double-stranded 32P-labeled si-inducing element probe (30,000 cpm) was incubated for 30 minutes at room temperature with 10 µg of nuclear proteins and 1 µg of poly(dI:dC)/dI:dC), and the products were subjected to electrophoresis.

Western Blot Analysis

Total proteins were prepared from the cultured VSMCs or pooled arteries after cuff placement (n=6 to 8 for each group), and Western blotting was performed as previously described.\(^5\)\(^10\) Immunoblotting was done using anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-STAT1, anti-phospho-STAT1 and 3 (New England BioLabs), anti-STAT3 (Santa Cruz Biotechnology), anti-phosphotyrosine, 4G10 and anti-Janus kinase (Jak) 2 (Upstate Biotechnology), Tyk2 and Rac1 (Santa Cruz Biotechnology), and anti-α smooth muscle actin antibodies (clone 1A4; Sigma). Densitometric analysis was performed using an image scanner (EPSON GT-8000) and National Institutes of Health imaging software.

Electrophoretic Gel Mobility Shift Assay

An electrophoretic gel mobility shift assay was performed as previously described.\(^10\) Briefly, the double-stranded 32P-labeled si-inducing element probe (30,000 cpm) was incubated for 30 minutes at room temperature with 10 µg of nuclear proteins and 1 µg of poly(dI:dC)/dI:dC), and the products were subjected to electrophoresis.

**Figure 1.** Effects of valsartan and/or fluvastatin on morphological changes after cuff placement. Morphometric analysis of neo-intima (a) and media (b) of femoral artery at 14 days after cuff placement. n=8 to 10 for each group. *P<0.05, §P<0.01 vs without valsartan and fluvastatin. Values are mean±SEM.

**Figure 2.** Effects of valsartan and/or fluvastatin on DNA synthesis after cuff placement. BrdU uptake in VSMCs in neointima (a) and media (b) of cuffed femoral artery 7 days after operation. n=8 to 10 for each group. *P<0.05, §P<0.01 vs without valsartan and fluvastatin. Values are mean±SEM.
Effects of Valsartan and Fluvastatin on Vascular Remodeling

We examined the effects of valsartan and fluvastatin on cuff-induced neointimal thickening. Valsartan (0.1 and 0.2 mg · kg⁻¹ · d⁻¹) and fluvastatin (1 and 3 mg · kg⁻¹ · d⁻¹) did not affect systolic arterial pressure or heart rate (data not shown), and fluvastatin did not influence plasma cholesterol concentration (control, 10.3±0.4 mg/dL; 1 mg · kg⁻¹ · d⁻¹ fluvastatin, 10.5±0.4 mg/dL; and 3 mg · kg⁻¹ · d⁻¹ fluvastatin, 10.2±0.3 mg/dL; n=6 to 7) after 14 days of treatment. Valsartan at 0.2 mg · kg⁻¹ · d⁻¹ or fluvastatin at 3 mg · kg⁻¹ · d⁻¹ decreased neointimal formation 14 days after the operation without a change in the medial area, and they decreased BrdU-positive VSMCs in the media and neointima 7 days after the operation (Figures 1 and 2). Valsartan or fluvastatin at lower doses (0.1 mg · kg⁻¹ · d⁻¹ or 1 mg · kg⁻¹ · d⁻¹, respectively) did not affect neointimal formation or the BrdU index, whereas coadministration of both valsartan and fluvastatin at these doses significantly decreased neointimal formation and BrdU index (Figures 1 and 2).

Cellular Mechanism of Inhibitory Effect of Fluvastatin on AT₁ Receptor–Mediated VSMC Proliferation

We examined the possibility that fluvastatin may inhibit AT₁ receptor–mediated VSMC proliferation. As shown in Figure 3a, pretreatment with fluvastatin (=5 μmol/L) for 24 hours inhibited Ang II (0.1 μmol/L)-induced [³H]thymidine incorporation dose-dependently in cultured rat VSMCs, which exclusively express the AT₁ receptor.⁹,¹⁰ Fluvastatin at 5 μmol/L inhibited the basal level of [³H]thymidine incorporation: pretreatment for 24 hours and additional stimulation for 36 hours with fluvastatin at 5 μmol/L and higher concentrations had a cytotoxic effect, regardless of whether Ang II was given, causing some cells to float. Therefore, it seems difficult to discuss the potential cross-talk of Ang II and 5 μmol/L fluvastatin in DNA synthesis in this experimental condition. Moreover, treatment with fluvastatin (0.1 to 5 μmol/L), which did not influence the basal level of c-fos transcription, inhibited Ang II (0.1 μmol/L)–induced c-fos

Statistical Analysis

Values are expressed as mean±SEM in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was found, post-hoc analysis was performed to detect the difference between the groups. Values of P<0.05 were considered statistically significant.

Results

Effects of Valsartan and Fluvastatin on Vascular Remodeling

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promoter activity (Figure 4a). This Ang II–mediated increase in [3H]thymidine incorporation as well as c-fos transcription was abolished by the addition of valsartan (10 μmol/L), but not by PD123319 (10 μmol/L), a specific AT2 receptor antagonist (data not shown). Treatment with orthovanadate (0.1 μmol/L) or okadaic acid (0.1 μmol/L) did not affect the inhibitory effect of fluvastatin on [3H]thymidine incorporation or c-fos transcription (Figures 3b and 4b).

Treatment with fluvastatin (0.1 to 5 μmol/L) for 24 hours did not affect the expression of the AT1 receptor (control, 8.78 ± 0.57 fmol/10^6 cells; 0.1 μmol/L fluvastatin, 8.89 ± 0.49 fmol/10^6 cells; 1 μmol/L fluvastatin, 8.68 ± 0.59 fmol/10^6 cells; and 5 μmol/L fluvastatin, 8.31 ± 0.63 fmol/10^6 cells; n = 4 to 5) determined by radioligand binding assay, whereas the AT2 receptor was undetectable by radioligand binding assay before and after fluvastatin treatment. To examine the signaling mechanism by which fluvastatin inhibited AT1 receptor–mediated VSMC proliferation, we focused on ERK activity, because the ERK pathway that is activated by the AT1 receptor and growth factors is critical for cell proliferation.12 As shown in Figure 5, pretreatment with fluvastatin (0.1 to 5 μmol/L) for 24 hours significantly inhibited the ERK activation induced by Ang II (1 μmol/L) stimulation for 15 minutes. Next we examined whether fluvastatin decreased AT1 receptor–mediated activation of STATs, because STATs seem to be involved in AT1 receptor–mediated c-fos expression in VSMCs.10 We demonstrated that pretreatment with fluvastatin significantly inhibited AT1 receptor–mediated tyrosine- and serine-phosphorylation of STAT1 and STAT3 after 15 minutes of Ang II treatment (Figure 5). Total protein levels of ERK, STAT1, and STAT3 were not changed.

Because we have reported that AT1 activation stimulates the binding of STATs with sis-inducing element in the c-fos promoter, resulting in increased c-fos expression, we examined the effect of fluvastatin on AT1 receptor–mediated DNA binding of the sis-inducing factor complex by electrophoretic gel mobility shift assay. As shown in Figure 6, pretreatment with fluvastatin inhibited AT1 receptor–induced formation of the sis-inducing factor complex. To analyze further the signaling mechanism of the inhibitory effect of fluvastatin on AT1 receptor–mediated STAT activation, we focused on Rac1, a small G protein, which has been shown to regulate Jak activation and Ang II–mediated cell proliferation.13,14 Cell lysates were immunoprecipitated with anti-Jak2 or anti-Tyk2 antibodies, and the phosphorylation of Jak2 and Tyk2 was examined by immunoblotting with an anti-

Figure 5. Effects of fluvastatin (Flu) on Ang II–mediated ERK phosphorylation and tyrosine- and serine-phosphorylation of STAT1 and STAT3. VSMCs were pretreated with fluvastatin (0.1 to 5 μmol/L) for 24 hours (a) or with fluvastatin (1 μmol/L) for 6, 12, or 24 hours (b) and then further stimulated with Ang II (0.1 μmol/L) for 15 minutes. Immunoblotting was done using anti-ERK, anti-phospho-ERK, anti-STAT1, anti-phospho-STAT1, anti-STAT3, and anti-α-smooth muscle (SM) actin antibodies. Figures show representative data from 5 separate experiments. p indicates phospho.

Figure 6. Effect of fluvastatin (Flu) on AT1 receptor–mediated sis-inducing factor (SIF) binding with sis-inducing element in VSMCs. Subconfluent and quiescent VSMCs were treated with or without fluvastatin (1 μmol/L) for 24 hours and then stimulated with Ang II (0.1 μmol/L) for the indicated times. Nuclear protein (10 μg) was incubated with [32P]-γ-ATP-labeled sis-inducing element (30 000 cpm) and analyzed by electrophoretic gel mobility shift assay. The results are representative of 3 experiments.
phosphorylation of Jak2 and Tyk2 was inhibited by fluvastatin treatment (Figure 7a). Ang II stimulation induced formation of a complex of Rac1, Jak2, STAT1, and STAT3 or Rac1, Tyk2, STAT1, and STAT3, and this Ang II–induced association of Rac1 with the Jak/STAT families was inhibited by fluvastatin (Figure 7b).

Consistent with these in vitro results, we observed that activation of ERK and tyrosine-phosphorylation of STAT1 and STAT3 were enhanced in response to vascular injury 7 days after operation and that administration of both fluvastatin (1 mg·kg⁻¹·d⁻¹) and valsartan (0.1 mg·kg⁻¹·d⁻¹) decreased activation of ERK, STAT1, and STAT3, without any change of total protein levels of ERK, STAT1, and STAT3, whereas fluvastatin or valsartan alone at these doses did not affect these parameters (Figure 8).

Discussion
Statins are effective in the prevention of end-organ damage; however, these benefits cannot be fully explained on the basis of cholesterol reduction. In the present study, we examined the possibility that statins may enhance the beneficial effects of an ARB on vascular remodeling. We demonstrated that treatment with even a low dose of valsartan together with a low dose of fluvastatin, which did not influence blood pressure or plasma cholesterol, effectively attenuated neointimal formation with a decrease in DNA synthesis in the media and neointima in an injured artery induced by polyethylene cuff placement, whereas these inhibitory effects were not observed with these doses of valsartan or fluvastatin alone.

Recent work points to additional cholesterol-independent effects of statins on cellular signal transduction. To elucidate the cellular and molecular mechanisms involved in the enhancement of the inhibitory effect of valsartan on vascular remodeling in vivo, we investigated the impact of statins on cultured VSMC proliferation. Exposure of VSMCs to Ang II–stimulated DNA synthesis and c-fos promoter activity via AT1 receptor activation and pretreatment with even a low dose of fluvastatin inhibited Ang II–induced DNA synthesis.
as well as c-fos promoter activity. ERK plays a role in VSMC proliferation, including the stimulation of c-fos transcription. Therefore, we examined the effect of fluvastatin on AT1 receptor-mediated ERK activation and observed that fluvastatin effectively inhibited ERK activation in VSMCs. Moreover, we observed that coadministration of even low doses of valsartan and fluvastatin effectively inhibited the phosphorylation of ERK in an injured artery in vivo. Consistent with our results, the AT1 receptor– and growth factor-mediated RAS/ERK pathway is efficiently inhibited by statins in human VSMCs, rat neonatal cardiomyocytes, and rat kidney.8,12,15

c-fos gene expression is regulated by the net interaction with different transcription factors. STATs are now known to be activated by many different extracellular signaling proteins including cytokines, growth factors, and Ang II via the AT1 receptor. We demonstrated that in response to AT1 receptor stimulation, tyrosine- and serine-phosphorylated STAT1 and STAT3 accumulated in the nuclei of VSMCs and became a component of the nuclear sis-inducing factor complex, resulting in enhancement of c-fos promoter activity.10 In the present study, we demonstrated that fluvastatin decreased tyrosine- and serine-phosphorylation of STAT1 and STAT3, as well as c-fos promoter activity in cultured VSMCs. The inactivation of ERK by fluvastatin may also result in decreased production of serum response factor, and this may act in concert with the inactivation of STATs via the inhibition of serine-phosphorylation,10 thereby resulting in a decrease of c-fos transcription. Consistent with these in vitro results, we observed that fluvastatin decreased the phosphorylation of STAT1 and STAT3 with valsartan. These results suggest that fluvastatin-mediated inactivation of STATs may contribute to the exaggeration of the inhibitory effect of valsartan on vascular injury.

Analysis of the detailed mechanism of the fluvastatin-mediated enhancement of tyrosine-dephosphorylation of STAT1 and STAT3 may provide further understanding of the inhibitory effects of statins on VSMC proliferation and atherosclerosis. It is less likely that fluvastatin activates and/or induces phosphatases, thereby inhibiting AT1 receptor–mediated signaling, because orthovanadate and okadaic acid did not influence the inhibitory effect of fluvastatin on AT1 receptor–mediated VSMC growth. Ang II–mediated tyrosine kinase Pyk2 activation in pulmonary vein endothelial cells has been reported to be inhibited by simvastatin through the inhibition of geranylgeranylation of the small G protein Rap1 and the subsequent association of Rap1 and Pyk2.16 It has also been reported that simvastatin inhibited platelet-derived growth factor–induced VSMC DNA synthesis, with inhibition of the geranylgeranylation of small G protein Rho,17 suggesting that small G protein Rho is involved in statin-mediated inhibition of cellular growth. Rac1, a member of the Rho family, has been shown to regulate the activation of Jak and STAT3.13 Recent evidence has indicated that Rac1 is involved in Ang II–induced VSMC growth.14 We observed that Ang II stimulation induced formation of a complex of Rac1 with Jak2, STAT1, and STAT3 or Rac1 with Tyk2, STAT1, and STAT3 and that this Ang II–induced association of Rac1 with the Jak/STAT families was inhibited by fluvastatin. Consistent with our observation, it has been reported that membrane translocation of Rac1 GTPase, which is required for the activation of NAD(P)H oxidase, was inhibited by atorvastatin in VSMCs in vitro and in vivo.18 Moreover, Takemoto et al19 reported that simvastatin inhibited cardiac hypertrophy and decreased myocardial Rac1 activity in rats treated with Ang II infusion or subjected to transaortic constriction. The inhibitory effect of fluvastatin on VSMC proliferation might be due to various alterations in cellular metabolism resulting from 24 hours of exposure to fluvastatin. The notion that Rac1-dependent regulation of Jak/STAT cascades could contribute to the inhibitory effect of fluvastatin on AT1 receptor–mediated actions in VSMCs is therefore an intriguing possibility.

Recent evidence has revealed that treatment with statins decreased AT1 receptor expression in VSMCs in vitro and in vivo.5,20 Moreover, it has been reported that statins downregulated AT1 receptor density in platelets.21 In contrast, Satoh et al16 reported that treatment of pulmonary vascular endothelial cells with simvastatin did not affect AT1 receptor expression. In our experimental conditions, pretreatment of VSMCs for 24 hours with a relatively lower dose of fluvastatin did not affect AT1 receptor expression. However, if relatively higher doses of fluvastatin would decrease AT1 receptor expression in VSMCs and the injured artery, we can expect more beneficial effects of statins combined with an ARB on vascular remodeling.

Whether these findings contribute to the effects of statins and ARB on atherosclerosis remains to be further investigated. The cholesterol-independent inhibition of AT1 receptor–mediated signaling and VSMC proliferation by statins demonstrates a novel regulatory mechanism that may contribute to the beneficial effects of these drugs beyond lowering of plasma cholesterol, especially together with AT1 receptor blockade by an ARB or ACE inhibitor. Our findings provide further insights into the pathogenesis of hypertension and atherosclerosis and may initiate rational and new therapeutic concepts.

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