Fluvastatin Prevents Vascular Hyperplasia by Inhibiting Phenotype Modulation and Proliferation Through Extracellular Signal-Regulated Kinase 1 and 2 and p38 Mitogen-Activated Protein Kinase Inactivation in Organ-Cultured Artery

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Objective—We examined the inhibitory mechanisms of fluvastatin on FBS-induced vascular hypertrophy assessed by organ-cultured rat tail artery.

Methods and Results—After 5 days of culture with 10% FBS, hyperplastic morphological changes in the media layer were induced. Treatment with 1 μmol/L fluvastatin significantly inhibited these changes. In the FBS-cultured arteries, the protein expression ratio of α-actin/β-actin was significantly decreased, indicating the change to synthetic phenotype. Fluvastatin restored the decreased expression ratio, and the addition of mevalonate (100 μmol/L) suppressed this recovery. In accordance with the synthetic morphological changes, the absolute force of contractions induced by stimuli was decreased. Fluvastatin treatment also restored the decreased contractility, and the addition of mevalonate suppressed this recovery. In the arteries cultured with FBS, extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (p38MAPK) phosphorylation were significantly increased. Fluvastatin inhibited these phosphorylations, and mevalonate prevented the action of fluvastatin.

Conclusion—These results suggest that fluvastatin inhibits vascular smooth muscle phenotype modulation to synthetic phenotype and proliferation by inhibiting the local metabolic pathway of cholesterol in smooth muscle cells, which inhibits hyperplastic changes in the vascular wall. The antihyperplastic actions by statins may be induced by inhibiting the ERK1/2 and p38MAPK activities, possibly through inhibition of prenylated Ras. (Arterioscler Thromb Vasc Biol. 2005;25:1-7.)

Key Words: fluvastatin ■ mevalonate ■ organ culture ■ smooth muscle hypertrophy ■ phenotypic modulation
ability to contract during hyperplastic responses. Recently, Sobue et al reported that the phenotype modulation of the smooth muscle cells is mediated mainly by a signaling pathway through mitogen-activated protein kinase (MAPK); growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor can activate extracellular signal-regulated kinase 1 and 2 (ERK1/2) or p38MAPK, which induces the phenotype modulation of smooth muscle cells from contractile to synthetic state. The authors further suggested that extracellular matrix is also important for maintaining the contractile state.

The vascular organ-cultured system has the distinct advantage of maintaining the differentiated contractile phenotype of smooth muscle cells because of better preservation of tissue architecture, cell-to-cell interactions, extracellular matrix, and cell morphology. In addition, this experimental system has enabled us to study the long-term pharmacological actions of external stimuli. Moreover, the vascular organ-culture method makes it possible to dissociate the influence of systemic cellular and humoral immune responses so that the direct effects of drugs on proliferation and the phenotypic change of smooth muscle cells can be investigated in a constant lipid concentration.

The aim of the present study was to gain an understanding of the pharmacological action of fluvastatin, an HMG-CoA reductase inhibitor, on the proliferation and dedifferentiation of VSMCs assessed by the vascular tissue organ-cultured method.

Materials and Methods

Tissue Preparation and Organ-Culture Procedure

Male Wistar rats were obtained from Charles River Breeding Laboratories at 8 weeks of age. Animal care and treatment were conducted in conformity with the institutional guidelines of The University of Tokyo and were consistent with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health.

The organ-culture procedure was performed as described previously. In all experiments, the endothelium was removed from the isolated tail artery. In the preparation for Western blot studies, adventitia was carefully removed as far as possible to avoid the contamination of fibroblast-like cells in the adventitia. The muscle rings placed in DMEM were maintained at 37°C under an atmosphere of 95% air and 5% CO2 for 5 days with or without 10% FBS.

Measurement of Muscle Tension

Muscle tension was isometrically recorded as described previously, with a force-displacement transducer (Orientec) connected to a strain amplifier (model 3134 or 3170; Yokogawa) under a resting tension of 5 mN. At the end of the experiment, papaverine (100 μmol/L) was added to determine the basal tone.

Morphological Examinations

Morphological examinations were assessed as described previously. The paraffin-embedded arteries were sectioned into 4-μm-thick sections, and the sections were stained with hematoxylin. To examine the proliferative change in vascular wall, the artery was cultured with bromo-deoxyuridine (BrdUrd; 20 μmol/L)–contained medium for 24 hours before fixation in the absence or presence of FBS. BrdUrd-positive cells were detected by using monoclonal anti-mouse BrdUrd antibody (DAKO) and stained by diaminobenzidine staining kit (StreptAB Complex/HRP kit; DAKO). Quantified BrdUrd-positive cells were shown as percentage of BrdUrd-positive cell number/total cell number in medial layer. Wall thickness was calculated as the diameter of the external elastic lamina minus the diameter of the internal elastic lamina.

Western Blot Analysis

The organ-cultured arterial tissues were homogenized in each suitable homogenizing buffer to extract each protein. Phospho-ERK1/2 and p38MAPK detection using Western blot was performed in accordance with the method described by Ikeda et al and Hayashi et al. The antibodies used were as follows: monoclonal mouse anti-human smooth muscle actin clone 1A4 (DAKO), monoclonal anti-β-actin clone AC-74 mouse ascites fluid, anti-ERK1/2 antibody, phospho-ERK1/2 antibody, p38MAPK antibody, and phospho-p38MAPK antibody (Cell Signaling).

Statistical Analysis

Results of the experiments were expressed as means±SE. Statistical evaluation of the data were performed by unpaired Student t test for comparisons between 2 groups and by 1-way ANOVA, followed by Dunnett’s test for comparisons between >2 groups.

Results

Morphological Changes of Vascular Wall

In the serum-free condition, the arterial wall maintained an intact morphology during the 5-day organ culture; the smooth muscle cells in the media layer were well arranged and had typical spindle-shaped nuclei (Figure 1A and 1B), as reported previously in the same tissue. However, in the medial layer of the 10% FBS-cultured arteries for 5 days, the smooth muscle cells were disarranged and degenerative, and round nuclei of the cells were observed (Figure 1C and 1D). These morphological changes were inhibited greatly by treatment with 1 μmol/L fluvastatin in the cultured medium containing 10% FBS (Figure 1E and 1F). Fluvastatin (1 μmol/L)
treatment for 5 days in the absence of FBS had no effect on the morphology, compared with the arteries cultured with serum-free medium alone (n = 9; data not shown).

On one hand, the hyperplastic change of the media layer observed in the FBS-treated tissue was significantly restored by treatment with 1/10000 mol/L fluvastatin (Figure 1E). On the other hand, the addition of mevalonate (100000 mol/L) to the cultured medium completely reversed the effects of 1/10000 mol/L fluvastatin (Figure 1G and 1H); smooth muscle cells were disarranged and degenerative, and round nuclei of the cells were observed, just like the arteries treated with 10% FBS alone for 5 days.

In Figure 2, the hyperplastic changes in the vascular wall were calculated by measuring the width in the media layer (Figure 2A) and internal diameter (Figure 2B). FBS treatment significantly increased and decreased the width of the media and internal diameter, respectively. Fluvastatin (1 μmol/L) almost completely restored the hyperplastic responses induced by FBS. However, fluvastatin (1 μmol/L) alone, added to the serum-free medium for 5 days, did not change the width of the media (77±5.4 μm in the serum-free treated arteries, n = 9; 77.1±4.2 μm in the serum-free with 1 μmol/L fluvastatin-treated arteries, n = 6) and internal diameter (295±17 μm in the serum-free treated arteries, n = 9; 260±13 μm in the serum-free with 1 μmol/L fluvastatin-treated arteries, n = 6). We further examined the effect the supplementation of mevalonate to the cultured medium with fluvastatin had on these parameters in the FBS-induced hyperplastic arteries. At a lower concentration of mevalonate (5 μmol/L), the decrease in internal diameter but not the increase in the width of the media was slightly restored. At 100 μmol/L, mevalonate completely inhibited the effects of fluvastatin on these parameters.

We further analyzed whether FBS-induced hyperplastic changes are mediated by proliferation or not. As shown in Figure 3A, BrdUrd-positive cells were detected in the medial layer of artery treated with FBS. The number of BrdUrd-positive cells in media layer was significantly greater in the artery treated with FBS than in the artery treated with DMEM alone. In addition, smooth muscle cell number was significantly increased in the FBS-treated arterial wall (Figure 3B). The increased cell number of the media layer treated with FBS was reduced by treatment with 1 μmol/L fluvastatin. The inhibitory effect of fluvastatin was suppressed by the supplement with 100 μmol/L mevalonate.

We next examined the effects of MEK inhibitor PD98059 or p38MAPK inhibitor SB203580 on the FBS-induced hyperplastic changes in medial layer. Each inhibitor was added into the cultured medium in the presence or absence of 10% FBS. Treatment of PD98059 (30 μmol/L) or SB203580 (20 μmol/L) alone did not inhibit the hyperplastic changes in the FBS-treated artery (Figure 2). However, combined treatment with PD98059 and SB203580 significantly inhibited these morphological changes.

ERK1/2 and p38MAPK Activation in the Cultured Artery

Morphological examination (Figures 1 through 3) revealed that fluvastatin inhibited hyperplastic changes in the media layer of the rat tail artery induced by FBS, indicating that statin may inhibit phenotypic and proliferative changes in smooth muscle cells. In addition, treatment with ERK and p38MAPK inhibitors inhibited the vascular hyperplastic...
Smooth Muscle Phenotypic Change by Monitoring Expression Ratio of α-Actin to β-Actin

We next examined expressions of the α-actin and β-actin proteins to assess the smooth muscle phenotype. In the 10% FBS-treated arteries for the 5-day culture, the α-actin content was decreased, and the β-actin content was increased, resulting in a decrease of the ratio of α-actin/β-actin as shown in Figure 5, indicating synthetic state of VSMCs. In the 10% FBS and 1 μmol/L fluvastatin-treated arteries for 5 days, the decreased ratio of α-actin/β-actin induced by the FBS treatment was recovered to the level of the serum-free control. In addition, supplementation of mevalonate (100 μmol/L) to the medium containing 10% FBS and 1 μmol/L fluvastatin decreased the expression ratio of α-actin/β-actin to the level of 10% FBS alone.

Smooth Muscle Contractility

In the serum-free control artery, morphology and contractile properties were well maintained for ≥5 days of culture compared with those in freshly isolated artery; the absolute force of norepinephrine (1 μmol/L)-induced contraction in the artery treated with DMEM alone (69.7 ± 8.1 mN/mg wet weight) did not differ from that in the freshly isolated artery (63.5 ± 7.3 mN/mg wet weight). On the other hand, in the 10% FBS-treated arteries for 5 days, the contraction induced by 1 μmol/L norepinephrine was reduced by ~60% compared with the serum-free control arteries (Figure 6A). In the 5-day cultured arteries with 10% FBS and 1 μmol/L fluvastatin, the FBS-induced inhibition was completely reversed. Similar inhibitory effects of FBS were observed when the muscle strips were stimulated with 100 nM endothelin-1 (Figure 6B) and 35 mmol/L KCl (high K⁺; Figure 6C), and 1 μmol/L fluvastatin significantly recovered the FBS-induced

Figure 4. Effects of fluvastatin (Fluva) on FBS-induced phosphorylation of ERK1/2 and p38MAPK at 5 days culture after stimulation with FBS. Results are expressed as means±SEM of 4 to 13 independent experiments. *Significantly different from FBS-treated artery; P<0.05. †Significantly different from FBS-treated artery; P<0.01. Mev indicates mevalonate.

As shown in Figure 4A, the amount of phosphorylated ERK1/2 was significantly increased by FBS treatment for 5 days (0.71 ± 0.10; DMEM alone 1.93 ± 0.17; FBS [P<0.01]; n=11 and 13, respectively). Fluvastatin (1 μmol/L) almost completely inhibited this phosphorylation (0.98 ± 0.13; n=4; not significant by different from DMEM alone). In addition, 100 μmol/L mevalonate treatment completely prevented the effects of fluvastatin; the amount of phosphorylated ERK1/2 was increased to a level similar to that in the arteries treated with 10% FBS alone for 5 days (1.75 ± 0.16; n=4). Farnesyl protein transferase inhibitor III (FPT III; 25 μmol/L) completely inhibited the FBS-induced ERK1/2 phosphorylation (0.97 ± 0.14; n=7; not significant by difference from DMEM alone), but mevalonate (100 μmol/L) treatment did not restore the inhibitory action by FPT III (1.05 ± 0.14; n=3). In addition, geranylgeranyl transferase inhibitor (GGTI; 20 μmol/L) had no effect on the FBS-induced ERK phosphorylation (1.84 ± 0.37; n=4).

We further analyzed the level of the p38MAPK phosphorylation in this vascular organ-cultured system (Figure 4B). After the culture of artery with FBS for 5 days, the level of p38MAPK phosphorylation was significantly increased (0.21 ± 0.01; DMEM alone 0.62 ± 0.06; FBS; n=6 each; P<0.01). Fluvastatin (1 μmol/L) treatment inhibited phosphorylation of p38MAPK (0.43 ± 0.04; n=6), and mevalonate (100 μmol/L) suppressed this inhibitory effect of fluvastatin (0.62 ± 0.05; n=6).

Figure 5. Effect of FBS, fluvastatin (Fluva), or mevalonate (Mev) on expression ratio of α-actin protein to β-actin protein. Top panel shows typical traces of Western blot analysis. Histograms show fluorescence intensity of bands expressed as the ratio of β-actin/α-actin. Results are expressed as means±SEM of 6 independent experiments. **Significantly different from serum-free; P<0.01.
In cultured VSMCs, it has been reported that statins inhibit cell proliferation, whereas some VSMCs. In organ-cultured system, typical medial hyperplasia was induced, however, failed to observe intimal hyperplasia, which is observed in vivo. In the present study, we found that ERK/p38MAPK and JNK signaling, which changes in the balance between phosphoinositide 3-kinase/Akt signaling and ERK/p38MAPK signaling, is also important for phenotype conversion from the contractile state to the synthetic phenotype. In the present study, we demonstrated that fluvastatin (1 μmol/L) drastically inhibited the hypertrophic changes in the vascular wall in cultures isolated on VSMC in culture. This action could be the only viable method of evaluating the action of fluvastatin on VSMCs, which is at a contractile form in the absence of FBS, this method could be the only viable method of evaluating the action of fluvastatin on VSMCs, which is at a contractile form in the absence of FBS. This antihypertrophic effect of fluvastatin was accompanied by an increase in the expression of mevalonate. In the present study, we found that ERK/p38MAPK and JNK signaling modulate the VSMC phenotype, and that the expression ratio of mevalonate significantly increased in the media layer. In conclusion, the present study demonstrated that fluvastatin has antihypertrophic actions, and that the expression of mevalonate significantly increased in the media layer. In conclusion, the present study demonstrated that fluvastatin has antihypertrophic actions, and that the expression of mevalonate significantly increased in the media layer.
mediated p38MAPK or JNK signal transduction is partially involved in vascular remodeling, indicating the possibility that JNK signaling may be also activated in the organ-cultured hyperplastic model. Further investigation will be necessary to clarify this point.

It is generally thought that smooth muscle phenotypic modulation to synthetic type is not essentially associated with cell proliferation. In fact, it was reported that growth factors, such as PDGF and EGF, which trigger VSMCs phenotype modulation to synthetic state, did not induce proliferation. However, others reported that the serum stimulation-mediated VSMCs phenotype modulation to synthetic state was associated with cell proliferation. Moreover, it has been suggested that cultured VSMCs in the synthetic state can proliferate only in the presence of FBS which contains mixed growth factors. These reports have led us to speculate that fluvastatin may inhibit not only the phenotype exchanging processes, but also the proliferation processes stimulated with FBS.

Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are important lipid attachments for the post-translational modification of Ras and Rho, respectively. In fact, it has been reported that the pharmacological control mevalonate pathway is effective for vascular smooth muscle growth and proliferation. In the present study, FPT III, a selective inhibitor of FPT, completely inhibited the ERK1/2 phosphorylation induced by FBS. In contrast, GGTT had no effect on ERK phosphorylation. Thus, in the organ-cultured rat tail artery, FBS stimulation mainly activates Ras but not Rho, Rac1, or other kinases, which resulted in activation of ERK1/2. Fluvastatin almost completely diminished the phosphorylation of ERK1/2 induced by FBS. Furthermore, supplementation of mevalonate to the cultured medium reversed these effects in the vascular organ-cultured system. These effects suggest that fluvastatin causes mevalonate starvation inside the VSMCs and that this, in turn, causes inhibition of lipid attachments for the post-translational modification of small GTP-binding proteins such as Ras; and finally, the FBS-induced phenotype modulation to synthetic state and proliferation of smooth muscle cells are inhibited by the reduced activities of ERK and p38MAPK.

In summary, fluvastatin inhibits FBS-induced VSMC phenotypic modulation through the Ras–ERK1/2 and p38MAPK signal transduction pathway, and thereby inhibits hyperplastic changes in the vascular wall and restores vascular function, as assessed by the vascular organ-cultured system. These effects may be mediated by the starvation of mevalonate after inhibition of HMG-CoA activity in smooth muscle cells.

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References


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